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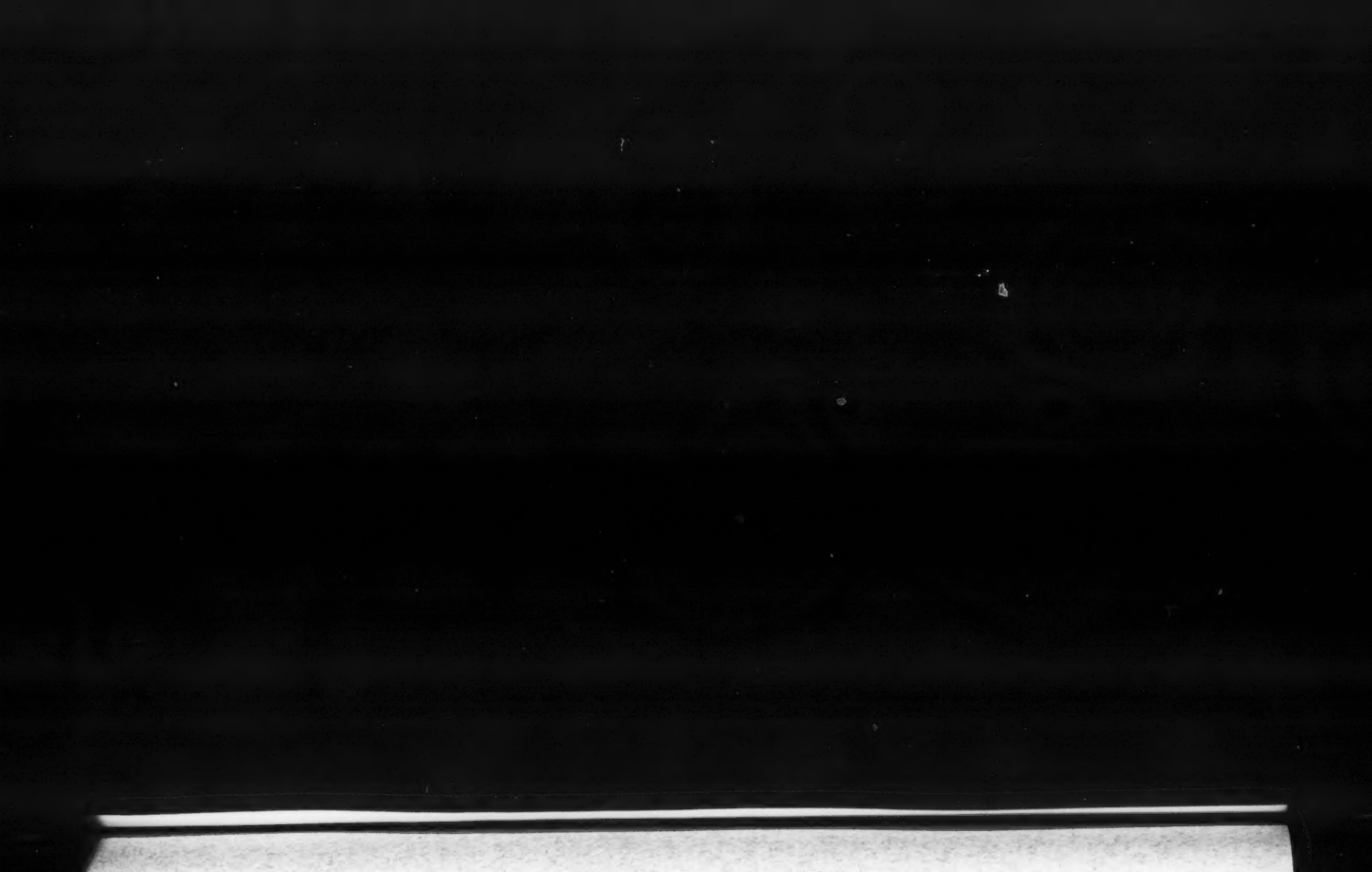

Vol. LII *April, 1927* *No. 4*

BIOLOGICAL BULLETIN

A LIPO-GEL REACTION EXERTED BY FOLLICULAR FLUID UPON SPERMATOZOA AND ITS SIGNIFICANCE (LILLIE'S REACTION).

GREGOR T. POPA.[1]

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I. INTRODUCTION.

Some time ago Professor F. R. Lillie observed that when sperm suspensions are mixed with follicular fluid taken from the Graafian follicles a coagulum is obtained (unpublished). As this phenomenon may be significant for the process of fertilization, Professor Lillie suggested that I study the reaction and determine the exact conditions under which it appears. I desire to express my thanks to Professor Lillie for suggesting this subject of research and for placing at my disposal the facilities of the zoölogical laboratories of the University of Chicago.

II. MATERIALS AND METHODS.

Several species were used in the study, chiefly cattle and sheep: Fresh organs obtained at the slaughter-houses of Swift and Company, Chicago,[2] were always employed. For comparison material was also taken from the goat, opossum, pig, and guinea-pig. The testes and ovaries were usually taken from

[1] From the Hull Zoölogical Laboratory and Whitman Laboratory for Experimental Zoölogy, University of Chicago.

[2] The author wishes to show his thanks to the officers of Swift and Co., who provided the material necessary for this work.

the bodies of animals just killed and carried to the laboratory in a thermos bottle. Under these conditions the organs remained at a temperature of 25°–30° C. until they were used. The experiments were almost always completed within two to four hours after the removal of the organs from the animals. To establish certain points which required absolutely fresh material, the same series of experiments was performed twice, once at the slaughter-house, within a quarter of an hour after killing, and again in the laboratory.

The spermatozoa were generally removed from different parts of the epididymis by transverse cuts through that structure. Sometimes they were also taken from the vas deferens and from the testes directly and also from the seminal vesicles. The follicular fluid was obtained by pricking the follicles at their most transparent points by means of a fine pipette into which the follicular fluid was drawn by suction.

All organs and especially the female genital tract were taken from the freshly killed animal by myself and examined immediately.

Other details of the technique will be described and explained in connection with the experiments.

III. Experiments.

1. When follicular fluid is mixed with spermatozoa taken directly from the epididymis or other parts of the male genital tract a coagulum is obtained in ten to twenty minutes. This coagulum is whitish, semi-opaque, friable, and of a specific gravity greater than that of water; when dissociated with needles, the coagulum breaks up into blocks with sharp edges, indicating a firm consistency. Small fragments of the coagulum when viewed under the microscope appear as homogeneous and transparent masses in which the spermatozoa are imbedded, scattered or in groups. After more complete dissociation of the coagulum into minute fragments one may isolate here and there bundles of fibers coated by a hyaline substance. The surface of the coagulum shines like a fatty surface and does not adhere to water. If therefore the coagulum is set carefully on the surface of water, it will float. When submerged, it sinks, showing that its specific gravity is greater than that of water.

This coagulum is produced at all temperatures between 10° C. and 56° C.; higher temperatures are unfavorable for the reaction. After its formation the coagulum persists at temperatures to 100° C. The fluid around the coagulum produces a second coagulum when heated to 72° C.

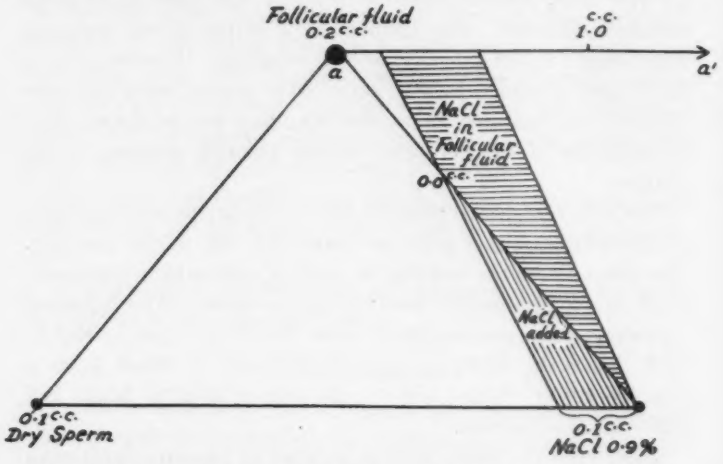
2. When follicular fluid is mixed with spermatozoa only, the results are not uniform. For example in one series of 90 tests, the result was negative in 43 cases, positive in 36 cases, and uncertain in 10 cases. But if the spermatozoa are mixed with 0.9 per cent. sodium chloride solution the reaction is more certainly obtained. For instance in a series of 242 tests, 41 were negative, 198 positive, and 3 uncertain. Furthermore in many cases in which the reaction failed to appear, using follicular fluid and spermatozoa alone, the coagulum was produced upon the addition of 0.9 per cent. sodium chloride solution to the mixture.

This result suggested that the salts which are normally present in the follicular fluid play an important rôle in the reaction. The effect upon the reaction of several salts which commonly occur in organic liquids was thereupon tested. The following salts singly or in mixtures were tried: NaCl, KCl, $CaCl_2$, $MgCl_2$, LiCl, $NaHCO_3$, $KHCO_3$, and $CaCO_3$. All of these gave a negative result. Only sodium chloride is able to favor and accelerate the reaction.

Since, however, there were a number of negative cases even after the addition of sodium chloride, an explanation for the failure of the reaction in these cases was sought. It was found that the reaction always occurs when the constituents are employed in certain definite quantities. After numerous tests, the following proportions were found to be successful in nearly all cases: 0.9 per cent. sodium chloride, 0.1 cc.; follicular fluid, 0.2 cc.; dry sperm, 0.1 cc. In a series of 128 tests using these proportions only four tests were negative.

The rôle of these proportions in the reaction may be illustrated graphically as in Fig. 1. If too much sodium chloride is added to the same amount of sperm, then the dilution of the sperm is too great and the reaction fails to appear. On the other hand if the quantity of sperm is excessive, then the action of the salt

solution upon the sperm—necessary for the reaction—is inadequate and the coagulum is not formed. With an excess of follicular fluid, the coagulum appears surrounded by a large quantity of fluid; if the quantity of follicular fluid is insufficient, the reaction fails also. From this we must conclude that some substance in the follicular fluid is essential for the formation of the coagulum. A minimum quantity of follicular fluid is thus necessary; this quantity (0.2 cc. to 0.1 cc. dry sperm) is indicated at the point *a* in the diagram. The line from *a* to *a'*



indicates an indefinitely increasing quantity of follicular fluid in which the reaction is still possible, providing the proportions of dry sperm and sodium chloride solution remain constant in the ratio of 1 : 1. We must assume also an active maximum quantity for the sodium chloride solution, namely, a quantity equal to that of the amount of dry sperm. From this amount the proportion of sodium chloride may be decreased indefinitely to zero, at which the reaction is still possible, providing the follicular fluid contains sufficient sodium chloride.

The four negative cases obtained with correct proportions stated require explanation. The following possibilities are suggested:

(*a*) Possibly the essential substance in the follicular fluid was insufficient in quantity or lacking altogether.

(*b*) Possibly the quantity of sodium chloride normally present in the follicular fluid was lacking or reduced so that even after the addition of 0.1 cc. sodium chloride solution the amount of this salt present was still insufficient.

(*c*) Possibly the proportions of the three substances used was not perfectly correct as they were measured by drops usually.

For all practical purposes the proportions of follicular fluid, sperm and sodium chloride solution stated above are sufficiently accurate and seldom fail to yield the reaction.

3. After the conditions necessary for the production of the coagulum had been determined, a new series of experiments was undertaken to establish more definitely the nature of the reaction. The results may be summarized briefly.

(*a*) The reaction is not produced by other liquids of the organism. I added follicular fluid or dry sperm to the following body fluids: amniotic fluid, blood taken from the jugular vein, defibrinated blood, smear of the mucous membrane of the uterus and tube, urine, cerebrospinal fluid, aqueous humor of the eye, vitreous body of the eye, peritoneal fluid, and pericardial fluid. In all cases the result was negative.

(*b*) The reaction is interspecific. The following combinations were tried:

Dry Sperm.	Follicular Fluid.
ram	× cow
bull	× sheep
goat	× cow
goat	× sheep
opossum	× cow
guinea-pig	× cow
ram	× pig

In all of these tests the result was positive.

(*c*) Materials from different sources. Follicular fluid taken from one follicle of the cow was tested with sperm taken from the epididymes of ten different bulls. The result was positive in all cases. Follicular fluid from ten different ovaries of the cow was tried with dry sperm taken from the same epididymis of a bull with positive results in seven cases only. Probably

some of the follicles had not reached the proper stage of concentration of the necessary constituents. However, follicular fluid taken from follicles of ten different sizes (cow and sheep), from the smallest to the largest gave a positive result in all cases. The age of the follicle does not appear to be of importance for the reaction.

(*d*) Time of reaction. In a series of ten tests (cow and sheep) observed after ten minutes, six were positive and four were negative. In ten tests observed after twenty minutes, all showed the coagulum. In a third series of ten tests, five of which were observed after one hour, five after 24 hours, all were positive. Thus the minimum average time necessary for the reaction lies between ten and twenty minutes. But the reaction is often observable after one minute. When dry sperm is mixed with follicular fluid on a slide, the reaction appears instantly. A delicate layer of coagulum is formed on the surface of the fluid.

(*e*) Variations in the time of addition of one of the components. In these experiments two of the three components were mixed and the third constituent added after a time interval, varying from one to twenty minutes. The following are the possible combinations:

(1) Follicular fluid mixed with salt solution; sperm added later.

(2) Sperm mixed with salt solution; follicular fluid added later.

(3) Sperm and follicular fluid mixed; salt solution added later.

No noticeable difference in the time of appearance of coagulum was noted in all three cases counting from the time of addition of the third constituent. In the third case the beneficial effect of salt solution on the reaction was again verified.

(*f*) Variations of temperature.

Dry sperm and follicular fluid were brought to various temperature and mixed (with the addition of salt solution as usual).

The results are summarized on opposite page.

These results show that there is in the follicular fluid a thermolabile substance which is responsible for the reaction. If the follicular fluid is heated to 56° C. the reaction fails. If the sperm is heated to a high temperature, even to 100° C. the reaction is obtained as long as follicular fluid is not heated above 55° C.

Follicular Fluid.	Result.	Dry Sperm.
10° C.	Positive	10° C.
28° C.	"	28° C.
37° C.	"	37° C.
40° C.	"	56° C.
45° C.	"	57° C.
50° C.	"	56° C.
54° C.	"	56° C.
55° C.	"	56° C.
56° C.	Negative	56° C.
28° C.	Positive	56° C.
28° C.	"	72° C.
56° C.	Negative	100° C.
56° C.	"	28° C.

(*g*) Freshness of material. Dry sperm and ovaries were kept in the refrigerator and used for the test at various intervals. It was found that sperm fifteen days old and follicular fluid eight days old still yield a positive reaction either when tested with each other or with fresh sperm or follicular fluid respectively. The age of the materials therefore within the limits stated does not affect the reaction.

4. In order to obtain a better understanding of the nature of the reaction follicular fluid and dry sperm have been examined microscopically and the literature has been consulted as to the possible constituents present in these two fluids. I found that dry sperm may contain spermatozoa, spermatocytes, blood, and lipoid substances. Follicular fluid contains or may contain blood (red and white corpuscles and many substances in solution or suspension), granulosa cells, proteins, sodium chloride and other salts, and pigment. I attempted to determine which of these constituents are involved in the reaction.

(*a*) Blood. It is very difficult to avoid the admixture of blood into either sperm or follicular fluid owing to the cutting or pricking of blood-vessels in obtaining these substances. In my earlier experiments blood was constantly present in my tests and I therefore was inclined to ascribe the reaction to the presence

of blood. Later, however, I took very minute precautions[1] to avoid admixture of blood and found that the reaction was always obtained in the complete absence of blood. I also while at the slaughter-house repeatedly mixed blood from the jugular vein with follicular fluid and never obtained the reaction.[2] It thus appears that the blood, and by implication the various constituents of the blood are not involved in the reaction.

(*b*) Proteins. A series of experiments performed with egg albumin gave completely negative results. This result would be expected from the negative finding with blood. Another proof that proteins are not involved may be derived from the fact that the reaction fails at a temperature of 56° C., although the fluid so heated and incapable of producing the reaction still contains proteins which do not coagulate until a temperature of 72° C. is reached.

(*c*) Sodium chloride. This salt and salts in general never produce the coagulum when mixed with either spermatozoa alone or follicular fluid alone.

(*d*) Granulosa cells. Follicular fluid was centrifuged and the clear fluid filtered to remove all cells. The absence of cells was verified by microscopic examination. The clear fluid was then violently agitated with glass granules for fifteen minutes with the idea of possibly altering the degree of dispersion of colloidal material that might be present. The fluid was then filtered through four sheets of soft filter paper. The fluid so treated still produces the reaction, indicating that cells or other formed constituents are not responsible for the coagulum. It may be mentioned here that filtered follicular fluid was always employed in all experiments.

(*e*) Spermatocytes. I was unable to separate the spermatocytes from the spermatozoa and therefore could not test their effect on the reaction separately. But I used material taken

[1] Ovaries taken directly from the body; washing them very carefully in laboratory before experiments; after that drying by absorbent paper; dissecting the follicles with a sharp knife until they appear like transparent vesicles. Only after that treatment puncture of follicles.

[2] In this case the anticoagulant effect of the sperm or of follicular fluid can be explained by the properties of NaCl and fatty substances, both able to stop the normal coagulation of the blood. Gilbert et Weinberg, "Traité du sang," 1913–1921, p. 15–16.

directly from the testes of animals of various ages and always obtained a positive reaction. Material from the testis may be supposed to contain a larger percentage of spermatocytes or cells in other stages of spermatogenesis than material taken directly from the epididymis or vas. No difference can be seen in the reaction however.

(*f*) Spermatozoa. Spermatozoa taken from any part of the epididymis, testis, vas deferens, or seminal vesicle always yield the reaction under the conditions previously described. Spermatozoa have been ground in a mortar for ten minutes, then mixed with salt solution, centrifuged, and filtered through four sheets of filter paper. The fluid obtained in this fashion was observed under the microscope; no spermatozoa or fragments of spermatozoa could be distinguished. Such a fluid however gives the reaction both with untreated follicular fluid and with follicular fluid that has been agitated, centrifuged, and filtered.

Thus all of the experiments to this point show that a coagulum is produced normally by a particular substance present in the follicular fluid, a product of the ovary, when mixed with products of the testes, especially the spermatozoa. The follicular substance is thermolabile and resistant to the action of mechanical factors. The experiments also show that the spermatozoa as formed cells are not essential to the reaction.

Up to this point in the experiments the reaction appeared to be perfectly specific: only products of the ovary act upon products of the testes.

5. Rôle of lipoids. Through certain microscopical observations, however, a new line of experimentation was suggested to me. Whenever spermatozoa treated with sodium chloride or with follicular fluid were studied under the microscope it was noted that after a short time the field of the microscope became filled with numerous small droplets, refractile, perfectly round, of the dimensions of cocci, and engaged in active brownian movements. The droplets increase in number with the degree of concentration of the salt solution.[1] When the suspension begins

[1] This phenomenon of drop production by action of salt solution is very clearly explained in Aschoff, L., "Zur frage der tropfigen Entmischung," *Verh. deutsch. path. Gesell.*, 17, Tagung München, 1914, p. 103–109. The influence of NaCl

to dry, the droplets run together into larger and larger drops which are also refractile and homogeneous. Their appearance is very similar to that of a fatty substance.

These droplets persist in normal hydrochloric acid but dissolve in sodium hydroxide (0.9 per cent.). When treated with a saturated solution of Sudan III. in 96 per cent. alcohol, all of the droplets turn red and appear as bright red points. These facts indicate the lipoid nature of the droplets.

The final analysis of this substance is a task for the chemist. The important fact in connection with these experiments is that the spermatozoa are always accompanied by a variable quantity of a lipoid substance.[1] This lipoid substance is responsible for the reaction with which this paper is concerned. The lipoid is in the state of a colloidal sol. When mixed with salt solution its state of dispersion is gradually changed but it still remains in the sol state. Upon the addition of follicular fluid, the thermolabile substance in this fluid exerts an influence upon the lipoid changed by salt solution and causes it to pass into the gel state. A coagulum is thus formed. Because of these characteristics of the reaction, I have designated the latter as a lipo-gel reaction.

The question to be next considered is whether the lipoid substance which surrounds the spermatozoa is specific for the reaction as is the thermolabile substance present in the follicular fluid. An article of Kölliker's suggested to me that the lipoid accompanying the spermatozoa might be similar in nature to myelin,[2] found in the central nervous system. I therefore mixed follicular fluid with a cell-free extract obtained by grinding up spinal cord (cow) in salt solution in a mortar and filtering. The

solution upon lipoids was observed also by Runnstrom, J., "Weitere Studien über die Verenderungen der Lipoide bei der befruchtung Seeigeleies," *Arch. Zool.*, 16, 1924, p. 1. The same effect was obtained instead of NaCl solution with thymol by Bidermann, W., "Über Wesen und Bedeutung der Protoplasma lipoide," *Pfluger's Archiv*, Bd. 202, 1924, p. 223.

[1] The same idea of a substance surrounding the spermatozoa is considered by Braus-Redenz and Redenz, H. E., Nebenhoden u. Samenfäden. Anat. Anz. Erg. 58, 1924, p. 121–131. But these authors merely postulate that such a substance exists and give no evidence concerning its nature. As regards the rôle of this hypothetical substance, the study of Redenz does not touch the essential meaning of it because the nature of the substance is not explained.

[2] A. Kolliker, "Phisiologische Studien über die Samenflüssigkeit," *Zeitschr. für wiss. Zool.*, Bd. VII., 1856, p. 201.

result was exactly the same as when follicular fluid is mixed with spermatozoa, except that the coagulum was more translucent.

Other organs which contain lipoid substances were then tested in the same way. The following were tried: thyroid, parathyroid, pancreas, adrenal gland, hypophysis, corpus luteum, skeletal muscle, heart, kidney, cerebrum, cerebellum, subcutaneous fat, subperitoneal fat, uterine mucous membrane, stomach, and lung. A positive reaction was obtained with all of these except the last three. The coagulum in all of these cases was quite similar and appeared under the same conditions as the coagulum produced by sperm and follicular fluid. Size, color, and time of formation vary a little with the organ used but the general result is the same.

Numerous experiments of this kind were performed; in each case tests were made in the slaughter-house with organs from freshly killed animals but without salt solution, and again in the laboratory in the manner described above. Sometimes when the reaction failed to occur when the tests were made at the slaughter-house, it succeeded in the laboratory after the addition of sodium chloride. Thus the reaction with different organs and follicular fluid has the same characteristics as the reaction between spermatozoa and follicular fluid.

Furthermore the typical reaction is again obtained upon mixing a commercial lipoid product kephalin (made by Armour and Company) with follicular fluid. Extracts which I have prepared from spermatozoa and medulla oblongata[1] likewise give the coagulum when mixed with follicular fluid, although the coagulum is rather thin. None of the extracts mentioned nor kephalin give any reaction with amniotic fluid, aqueous humor of the eye, or hydatic fluid.

The reaction in all of these cases invariably fails to occur if the follicular fluid is heated to 56° C.

Thus the supposition that the coagulum results from the action of a particular thermolabile substance in the follicular

[1] Spermatozoa or ground nervous tissue were kept 24 hours in 96 alcohol, than 24 hours more in ether; evaporation on water bath. The residue dissolved in salt solution 0.9; centrifugation for elimination of cells themselves. The filtrates used for tests. *Cf.* Loewe, S., "Zur physikalische Chemie der Lipoide, I.–IV., *Biochemische Zeitsch.*, Bd. 42, 1912.

fluid on the lipoid material surrounding the spermatozoa is demonstrated to be correct.

6. The question next to be considered is the rôle of sodium chloride in the reaction. It has been shown that sodium chloride accelerates the reaction. Microscopical observation of the action of sodium chloride on spermatozoa indicates that the sodium chloride causes the minute droplets of lipoid to aggregate into larger droplets. We can conclude that the sodium chloride prepares the way for the action of the follicular fluid by producing some change in the colloidal state of the lipoid. Further research would be required to elucidate this point.

7. An additional point has been determined,—namely, that the lipoid substance occurs on the surface of and between the spermatozoa. This is shown by the following experiments:

(*a*) When spermatozoa are washed once with 0.9 per cent. salt solution and filtered, both the washed spermatozoa and the filtrate produce the reaction in five to ten minutes.

(*b*) When such spermatozoa are washed a second time and filtered the washed spermatozoa and the filtrate from the second washing yield the reaction only after 24 hours.

(*c*) The same spermatozoa after being washed a third time do not give the reaction in 24 hours nor does the filtrate from the third washing yield any coagulum in this time.

(*d*) These spermatozoa after being washed three times were ground in a mortar with salt solution and filtered again. The reaction again failed.

From these experiments it appears clear that the lipoid substance is not contained inside of the spermatozoa but occurs on their surfaces and between them. The question of the rôle of this lipoid for the life of the spermatozoa and in the process of fertilization will be the subject of new researches now under way.

IV. The Physiological Significance of Lillie's Reaction.

The first point to be considered is whether the reaction is organ specific. The experiments show that only follicular fluid produce the reaction and so it is organ specific in this sense. *Stricto sensu*, the reaction is however not specific for I have

shown that it can be obtained with products from a large number of organs and tissues. But as the reaction occurs in nature it may be said to be specific, for the follicular fluid under natural conditions does not meet any other fluid with which it produces a coagulum except spermatozoa. It has been shown that the reaction does not occur with peritoneal fluid, smear of the mucosa of the genital tract, or urine. The reaction is produced with lipoids but the only lipoid with which the follicular fluid would ordinarily come in contact is that accompanying the spermatozoa.

We may next discuss the possible physiological purpose of this coagulation phenomenon. At first sight the phenomenon appears to be of a paradoxical nature. In order to perform their function of fertilizing the egg the spermatozoa must retain their motility when introduced into the genital tract; yet it appears from my experiments that in the genital tract they meet a fluid, namely, the follicular fluid, which destroys their motility.[1] Without discussing this matter at too great length the following explanations of the utility of the reaction may be suggested.

1. Lillie has shown that the eggs of certain invertebrates contain and produce a substance which he designates as fertilizin which has the property of activating and agglutinating the spermatozoa and is of importance in the fertilization of the egg. If fertilizin is produced by the mammalian egg it would certainly occur in the follicular fluid. I could not determine the place of origin of the substance in the follicular fluid which gives the coagulating reaction with spermatozoa. It might originate from the ovum, or the granulosa cells. My experiments (p. 230) indi-

[1] It is very surprising that in the whole literature one can not find the slighest indication of this phenomenon. A tremendous number of workers have tried the reaction upon spermatozoa of different kind of substances *excepting* the follicular fluid and this seems the most logical substance to be tried. Professor Lillie was the first to try this reaction and thus opened an entirely new field of experimentation. The origin of the whole question concerning the lipo-gel reaction is included in the long series of works published by Professor Frank R. Lillie. Compare, *e.g.*, "Studies in Fertilization. V. Mechanism of Fertilization in *Arbacia*," *The Journ. of Exp. Zoöl.*, Vol. 16, 1914, p. 523; "Studies on Fertilization. V. The Behavior of the Spermatozoa of *Nereis* and *Arbacia* with Special References to Egg-extractives," *The Journ. of Exp. Zoöl.*, 1913, Vol. 14. As a general review of the entire problem in which my work must be integrated see Frank R. Lillie, "Problems of Fertilization," Univ. of Chicago Press. One can find there also a very rich literature concerning the subject.

cate that the granulosa cells (including presumably the ovum) do not contain sufficient of the substance to give the reaction. It was also shown that blood does not yield the reaction. It is therefore necessary to suppose that the substance responsible for the reaction is a modified product of either the blood or the granulosa cells plus the ovum, or of both, and does not exist as such in any of these objects; or that it is a substance which gradually accumulates in the follicular fluid. In favor of the hypothesis that the active substance originates from the cells of the follicle including the egg is the observation that follicular fluid from atretic follicles does not yield the reaction. The ovary of the cow frequently contains follicles filled with a clear colorless liquid; such follicles do not contain any granulosa cells or any ovum. They are readily recognized by the colorless watery appearance of the contained fluid. This fluid however invariably fails to yield the coagulating reaction, eleven cases having been tested.

The active substance in the follicular fluid does not appear to be identical with the fertilizin of Lillie in its properties. For instance, fertilizin is stated to be very resistant to heat, while the follicular substance as shown above loses its coagulating property when heated to 56° C.

2. Whatever may be the rôle played by the coagulating substance in the process of fertilization I believe that it may have another secondary function which does not necessarily exclude the first suggestion. I am inclined to think that the follicular fluid may serve to protect the peritoneum against possible infection by infected spermatozoa.

I have made a number of observations on the life and behavior of spermatozoa in various media and particularly in follicular fluid. From these experiments it appears that the spermatozoa furnish a very good medium for the growth of bacteria. After standing for three or four hours at room temperature sperm suspensions were seen to contain many cocci and bacilli: and after twenty-four hours they were swarming with microörganisms. For this reason spermatozoa live much longer at low than at higher temperatures. Spermatozoa will live for several days in small glass tubes if they are kept in the refrigerator, but only for

24 to 30 hours at room temperature. On two occasions spermatozoa that had been kept in the refrigerator for nine days showed motility when brought into room temperature.[1] Spermatozoa removed and kept under aseptic conditions may remain alive for eight days in the refrigerator and for four or five days at room temperature. Even infected spermatozoa retain their motility for some time and move through the field of the microscope carrying bacteria with them. I have injected spermatozoa from the ram into the excised uterus of a sheep. In two hours the spermatozoa were found to have penetrated into the uterine tube but they were greatly damaged and fragmented and many of them had been phagocitized. Those spermatozoa which were still alive were in a state of agitation and covered with bacteria. It appears that the surface of the spermatozoa is very sticky and that all kinds of particles therefore adhere readily to them.[2]

The spermatozoa are placed normally in a very infected organ, namely, the vagina. The penis itself bears some species of bacteria and at all events at the moment of copulation many bacteria are introduced from the outside into the vagina. These bacteria are carried up into the uterine tubes by the spermatozoa and constitute a menace for the peritoneum which is well known to be exceedingly sensitive to infection. The agglutination of the spermatozoa by the follicular fluid would hold these bacteria and permit them to be phagocytized more readily, thus protecting the peritoneum from possible infection.

This hypothesis of the protective rôle of the coagulating substance in the follicular fluid does not exclude the possible importance of this fluid in fertilization; also it is not possible, at the present time, to estimate more accurately the practical efficacy of this protective rôle of the coagulating substance.[3]

[1] The same observation on favorable effect of low temperature upon longevity of spermatozoa has been repeatedly made *e.g.* by Redenz in the paper already mentioned; *Cf.* also Mettenheimer, M., "Sperma und kunstliche Befruchtung bei Mensch und Tier," *Münch. Med. Woch.*, schr. 72, Jahr. g. 1925, p. 977; Mettenheimer, M., *Arch. f. Gynak.*, 1925, Bd. 162, p. 215.

[2] I found after I finished my work, that the proof that spermatozoa may transport bacteria was already furnished by Rotter, "Wie ascendiert die Gonorrhöe," *Archiv f. Gynäkologie*, Bd. 117, S. 153. This author has proved that spermatozoa can transport *Bacillus subtilis* and *gonococcus*.

[3] I am obliged for editing this paper to Dr. Libbie Hyman to whom I express my best thanks.

THE DISTRIBUTION OF SUBSTANCES IN THE SPERMATOZOÖN (*ARBACIA* AND *NEREIS*).

Studied by Intra Vitam Stains and by Stains of Lipoids According to the Method of Schumacher.

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I. INTRODUCTION.

In a previous paper [2] I come to the conclusion that the spermatozoa of mammals are surrounded by a layer of a lipoid substance which is responsible for a reaction observed by Prof. Frank R. Lillie, and called by me the *lipo-gel reaction*.

With other methods and in different animal species, I have been able to analyze much more completely the distribution of this lipoid substance in and between spermatozoa. The following description is a record of these new facts.

In a recent paper Josef Schumacher has devised methods for the identification of lipoids, lipoproteids and fats in the tissues and cells by means of combining different strong reagents with the action of stains. One conclusion of his work is that there is a series of stains (above all Victoria blue and fuchsin) which

[1] From the *Marine Biological Laboratory, Woods Hole, Mass.*

[2] Gregor T. Popa, 1927, "A Lipo-gel Reaction Exerted by Follicular Fluid upon Spermatozoa (Lillie's Reaction) and Its Significance, Biol. Bull., Vol. 52, p. 223.

have a special affinity for lipoids, giving a salt by combination with the lipoidic acid. After destroying the proteins by different macerating reagents, one can isolate the lipoids and in this case the staining with the mentioned substances is a specific stain.

I shall not review this method in detail. Everyone who needs to use it must read the original paper: Josef Schumacher: "Zur Chemie der Zellfaerbung VIII. Mitteilung. Ueber die Nachweis der Lipoide in Zelle und Gewebe," Chemie der Zelle und Gewebe, *Zeitschr. f. die Probleme der Gaerhung, Atmung u. Vitaminforschung*, Bd. XII., Heft 5, 1926, S. 433.

I have used his methods (see page 247) but most of the observations were made by simpler methods, using stains recommended by him as specific stains, and in addition various intra vitam staining methods. In the latter case the staining was done under the cover slip as in the method of Koltzoff "Studien ueber die Gestalt der Zelle," *Arch. f. Zellforschung*, Bd. II., S. 1, 1909.

II. RESULTS.

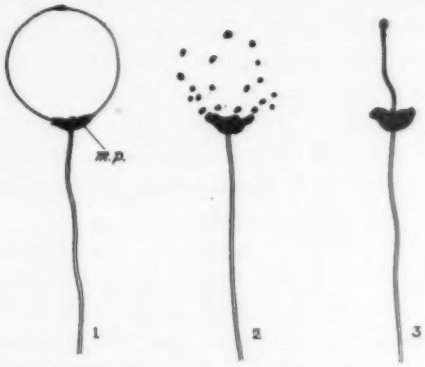
1. The Use of Stains with Fresh Spermatozoa.

(*a*) *Victoria Blue and Fuchsin.*

Victoria blue in 1 per cent. solution in distilled water: Stain under the cover slip: one drop of sperm suspension (one drop dry sperm to 10 cc. sea-water) is placed on the slide, and after covering with a cover slip, with a fine pipette one adds at the margin of the cover slip a drop of staining solucion. The fluid penetrates by capillarity, advancing more and more slowly. In this way, one gets in the same preparation all degrees of staining, from the strongest color to the complete lack of stain. At the one end of the slide the spermatozoa are overstained, and on the opposite side the spermatozoa are still active.

The first impressive fact is a remarkable change of the shape and size of the spermatozoa. The stain is in distilled water and the spermatozoa are in sea-water: there is a great difference in the osmotic pressure of the two fluids. Thus, a rapid penetration of the staining solution takes place into the spermatozoa, which stain immediately. Shortly after, the heads of the spermatozoa swell, become round and then burst; the following

diagrammatic figures show some of the variations in distribution of the stain (Figs. 1–3).



FIGS. 1, 2, 3. Fresh spermatozoa of *Arbacia* stained with 1 per cent. solution of Victoria blue in distilled water.

When the reaction is too strong, *i.e.*, when the contact of the spermatozoa (in sea-water) with Victoria blue (in distilled water) is immediate, the tails swell also from place to place and one may find shapes as in Figure 4. Frequently, when the reaction is still stronger, the tails twist round the heads and shapes very similar to those described by Koltzoff result (*loc. cit.*, pp. 9–12).

Victoria blue, fuchsin, and the dyes from the same series (Gentian violet, Malachite green, etc.) always stain substances in the acrosome region, in the middle piece, and in the tail. The reaction with Victoria blue, and with the stains in the same class, gives strong reasons for postulating the presence of lipoids in the composition of the acrosome, middle piece, and tail.

Victoria Blue in Alcohol (1 cc. of the distilled water solution in 10 cc. alcohol, 70 per cent.): The solution fixes the spermatozoa at the same time that it stains, and the stain being more dilute, one can see the gradual transformations of the spermatozoa. The heads swell slowly; the tails never swell. The distribution of the stain in the spermatozoa is exactly the same; tip, middle piece, tail. Also, the stain in the region of the acrosome does not always occur, and the middle piece varies in size. By variations of concentration one can have a series of pictures

more and more similar to those obtained by Victoria blue in distilled water.

Fuchsin (1 per cent. distilled water solution): The affinity of the fuchsin for the lipoids is less than that of Victoria blue.

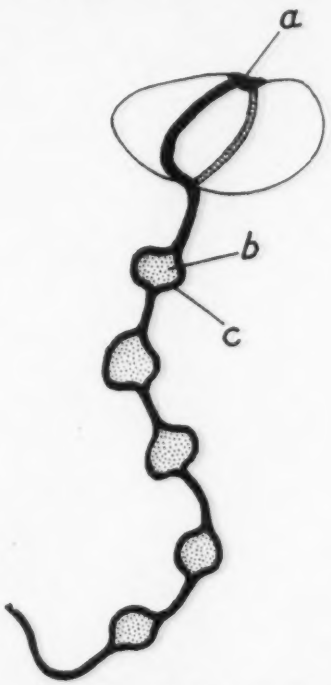


FIG. 4. *Arbacia.* Fresh spermatozoön in 1 per cent. Victoria blue in distilled water—Swelling of tail and twisting around head. *a*, middle piece; *b*, hydrophilic substance; *c*, lipophilic substance.

The staining is accordingly slower and one can follow this process more gradually in the parts of the slide where the effect of distilled water is moderated by a suitable mixing with sea-water.

The new fact obtained by this method is the possibility of observing *changes of shape and variations of size and form of the middle piece.* One can see in its region only granules varying in number (2–6), or a ring varying in size from one spermatozoön

to another, and in the same spermatozoön during the observation [1] until at the end of the reaction almost all spermatozoa show the middle piece as a compact mass, red-stained.

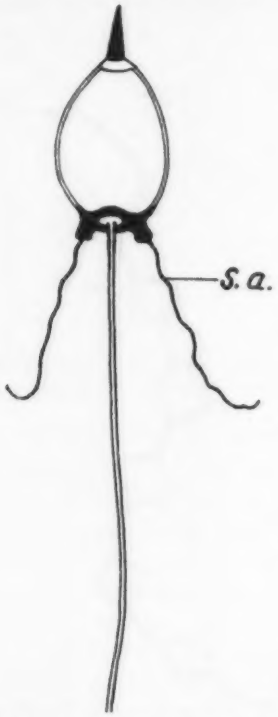


FIG. 5. Spermatozoön of *Nereis* to show sensillæ amœboideæ.

When the slowly moving spermatozoön shows its tip, one can see that there is really a minute opening. The tip of the spermatozoön is extremely sticky and adheres to everything

[1] The instability of this region was very clearly observed by Retzius and Ballowitz, but they believe that it occurs because of the technique. However, without staining, under conditions approximately normal, the same variations of shape can be noticed. Gustav Retzius, "Die Spermien von *Aurelia aurita* L.," *Biol. Unters.*, N. F., XIV., 1909, S. 67. Gustav Retzius, "Die Spermien der Nereiden," *Biol. Unters.*, N. F., XIV., 1909, S. 69. E. Ballowitz, "Über die Körnige Zusammensetzung des Verbindungsstückes der Samenkörper der Knochenfische," *Arch. f. Zellforschung*, 14 Bd., 1917, p. 355.

which touches it: granules, slide, cover slip, eggs, or to another spermatozoön. Frequently it sticks to the tail of the same spermatozoön.

Victoria blue or *Fuchsin* in sea-water solution: These substances are very feebly soluble in sea-water; but if we keep an excess of stain for several hours in sea-water, a weak solution is obtained in which the spermatozoa can live for ten to thirty minutes and one can see the changes which occur more easily than in distilled water solution.

FIG. 6. Spermatozoön of *Arbacia* showing sensilla amœboidea.

The regions of the spermatozoön already mentioned take the color. But for a short time there is a slight stain on the surface of the head also, before the middle piece is stained. After a variable time the head loses the color gradually and one can see a slow enlargement of the middle piece and the formation of delicate protoplasmic filaments therefrom, which move sporadically. These deserve a more precise description.

The basal part of the head has the appearance of a ring, somewhat thicker than the rest of the head. When the sperma-

tozoön turns in the fluid in such a manner as to show its larger end, one can see very clearly even on fresh preparation, not stained, the presence of this ring. It has different sizes, according to the species (*Arbacia* and *Nereis*) and different outlines on the same spermatozoön. Here and there it is thicker or thinner; sometimes it becomes fragmented in granules and again, it forms a compact mass. In *Nereis* (Fig. 5) there are normally two swellings placed symmetrically, outgrowths of the ring, on which one can distinguish two long filaments as in Fig. 5. In *Arbacia* (Fig. 6), the ring shows in the same way various thickenings (3–5) and these, not so constantly as in *Nereis*, support (usually only one of them) a prolongation ended by a kind of knob.

As the spermatozoön floats in the fluid, these filaments show protean movements, elongation, shortening and thickening, very similar to amœboid movements. They are constructed of the same material as the basal ring and they are to be considered as prolongations of this. Provisionally they may be called *sensillæ amœboideæ*.[1] In all changes of osmotic pressure, in all media which change or kill the spermatozoa, these sensillæ retract upon the ring where they produce granules projecting a little above the general level.

(*b*) *Janus Green; Trypan Blue; Neutral Red* (*1 Per Cent. in Sea-water*).

These substances are frequently used for staining intra vitam. The penetrating power is different for each. Janus green gave

[1] It is very interesting that the presence of these filaments was noticed by Mischer "Die Spermatozoen einiger Wirbeltiere. Ein Beitrag zur Histochemie," *Verh. der naturf. Gesellsch. in Basel*, 1878, Bd. VI.; the interpretation of them was wrong. E. Ballowitz, in the same species, denies Mischer's observations "Über die Samenkörper des Lachses. Ein weiteres Beitrag zur Kenntnis der Spermien der Salmoniden," *Arch. f. Zellforschung*, 14 Bd., 1917, S. 451; but in an older paper and in other species, "Untersuchungen über die Struktur der Spermatozoen, zugleich ein Beitrag zur Lehre von feineren Bau der Kontraktilen Elemente. Die Spermatozoen der Insekten," *Zeitschr. f. wiss. Zoöl.*, 1890, Bd. 50, S. 317, he himself has observed such filaments and even their movements. He even calls them *Wimpelfasern*. The meaning of them is not explained, and he says that they are "*rätselhaft*." The conditions of observation were bad because he employed always "maceration," after killing the spermatozoa. In fact, the sensillæ are to be observed much more clearly on fresh, slowly motile spermatozoa, without stain.

the best results in our experiments. It penetrates very slowly, and shows excellent contrasts.

By these stains one obtains more clearly the same results as by Victoria blue in sea-water. With Janus green especially, one obtains an entire series of pictures, which prove clearly that *there is a migration of the same substance which stains in Victoria blue*, to the connecting region between head and tail and a large accumulation there of the migrating substance (Fig. 7 *a–e*). But here and there one can find spermatozoa in which

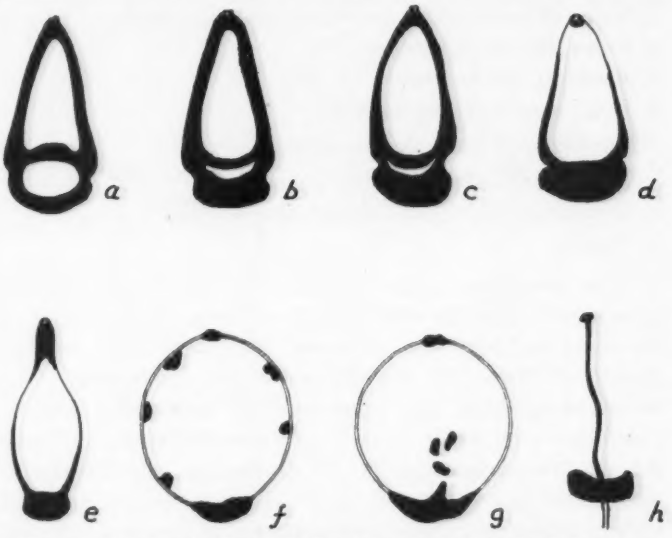


FIG. 7. *a–e*, sperm heads of *Arbacia* showing stages of staining in Janus green in sea-water; movements of lipochromatic substance. *f–h*, Victoria blue in sea-water, various conditions. The lipochromatic substance is shown black.

pare the action of Victoria blue and fuchsin in distilled water (Figs. 2, 3, and 4) in which one sees the lipochromatic substance also accumulated on various regions of the head. These facts show the presence in the spermatozoön of *an active cytoplasmic layer*, external to the nucleus, *loaded with lipoids* and *taking* the lipochromatic substance[1] migrates towards the apex. Com-

[1] I use this term *lipochromatic substance* for a succinct description, implying only that the substance mentioned takes very strongly the stains reputed by the work of *Schumacher* to be lipophiles: Victoria blue and fuchsin especially.

various forms. This layer flows in and out of middle piece and forms the sensillæ.[1]

2. Staining Methods after Fixation.

Spermatozoa of *Arbacia* and *Nereis* were also studied by the more usual methods of fixing and staining. The fixing methods used were:

1. Corrosive sublimate, saturated solution in distilled water, 100 parts, plus glacial acetic acid, 8 parts.
2. Vapor of osmic acid, 2 per cent. aqueous solution.
3. Flemming, strong solution.
4. Formalin, 10 per cent.
5. Heat, after rapid evaporation.

The following stains were employed:

1. Iron-hæmatoxylin (Heidenhain) and eosin.
2. Iron-hæmatoxylin (Heidenhain) and safranin.
3. Fuchsin.

These procedures applied to the study of the spermatozoa, when united with the observations on living material, give us the conviction that *there is no one fixing fluid which preserves perfectly the form and the structure of the spermatozoa*.[2] The fastest acting fixing agents (as vapor of osmic acid) and the most penetrating fluids (as acetic acid plus *k*-bichromate) change the size, the configuration of the spermatozoa and the mutual relations of the substances in it. The outlines of the head slightly change, the heads swell, the sensillæ disappear, the ring changes its shape, and the lipochromatic substance migrates.

[1] I have strong reasons to believe that Ballowitz has observed also the migration of the peripheral substance of the spermatozoa, "Untersuch. über die Struktur der Spermatozoön der Fische. Amphibien, Reptilien," *Arch. f. mikr. Anat.*, 36 Bd., 1890, S. 225. His explanation is not satisfactory. He believes that the change of color in various parts of the head of the spermatozoön is a consequence of stain diffusion in the medium (p. 239). But the changes can be better observed by the method of Victoria blue, fuchsin and Janus green than by his method; and one can also observe the accumulation of the substance in the connecting region even on spermatozoa not stained at all.

[2] Students of spermatozoa note the same thing. This fact is demonstrated particularly clearly by C. Pictet, "Rechêrches sur la spermatogénèse chez quelques inverté brés de la Méditerranée," *Mitteil a. d. Zoöl. St. Neapel.*, 10 Bd., 1891–93, S. 75.

3. Schumacher's Methods.

A.

One makes a smear with sperm as thick as possible, and before the sperm is dried the slide is plunged into HCl solution 1 : 4. The slides remain in the acid 24 hours. After that time, wash in distilled water and stain for 5 minutes in 1 per cent. Victoria blue in distilled water. Again 5 minutes in distilled water. Mount after usual treatment in cedar oil, or, in order to have clearer pictures (but more unstable) one can mount directly in water and seal with paraffin.

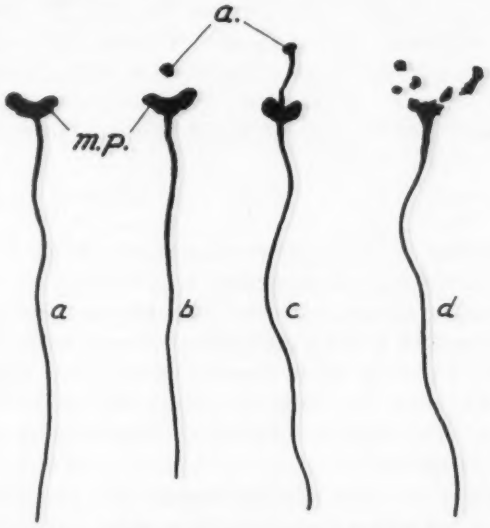


Fig. 8. Spermatozoa of *Arbacia* prepared according to method A of Schumacher and stained in Victoria blue.

The idea of this treatment is as follows: The various chemical components of cells show a different resistance to the various chemical agents. By combining the destructive agents and the time of acting, it is possible to isolate by turn the chemical components of the cell and detect them afterwards.

Schumacher uses strong acids in order to destroy the proteins of the cell. The remainder, after maceration, must be some

substance not influenced by acids, essentially lipoproteins (proteins united with lipoids) and lipoids. If this is true, the stains characteristic for proteins, such as methylen blue, will stain no more, and on the contrary, the stains of the fuchsin series still will give a coloration.

This method was used by Schumacher in studying leucocytes, yeast cells, liver, lung, and skin. I have applied this method in studying the spermatozoa.

With the above method one obtains with Victoria blue the following pictures (Fig. 8); staining with methylene blue fails completely. This demonstrates that the various portions of spermatozoa stained with Victoria blue by the procedures already described, resist the action of strong acids, while the other portions are destroyed. Therefore, according to the theory of Schumacher, we can say that *in the construction of the tail, middle piece, and acrosome of the spermatozoön there is a great quantity of lipoid substance.*

B.

For deciding if in an organic construction, which resists the action of acids and still is stained by Victoria blue, there are free lipoids or lipoids associated with proteins (lipoproteins), Schumacher uses another procedure: Smears made as in *A* (above) are placed for 24–36 hours into a mixture of alcohol and ether. Afterwards the slides are transferred for 24 hours to HCl 1 : 4. The staining following is identical with that described in *A* (above).

The idea is this: The alcohol-ether dissolves and washes out *free* lipoids, but does not touch the lipoproteids; afterwards HCl destroys the proteins, and again the lipoproteids remain intact, and thus they are isolated. If, after such a treatment, we still obtain a coloration, we can say that there is a lipoprotein component.

Applied to the study of spermatozoa, this method gives the pictures shown in Fig. 9. A pale reticulum built by interlacing tails, and here and there some knobs at the ends of fibers, inferred to be the remains of middle pieces. It is to be noticed also that the fresh preparations immediately after staining with Victoria

blue, are much clearer than the preparations mounted in balsam, and particularly after treating with xylol.

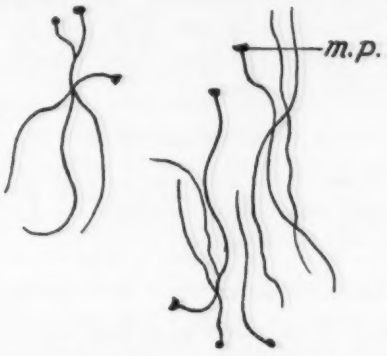


FIG. 9. Spermatozoa of *Arbacia* prepared according to method B of Schumacher and stained in Victoria blue.

Summarizing the facts obtained by the method of Schumacher, one can say that in the structure of the spermatozoön there are free lipoids and lipoproteids which are located normally in the tail, middle piece, and acrosome. The facts described in 1 and 3, connected with the facts observed in living spermatozoa, demonstrate that the lipoids and lipoproteids of the tail are located *on the surface.*

4. OSMIC ACID (1 PER CENT. AQUEOUS SOLUTION 24 HOURS, OR 2 PER CENT. AQUEOUS SOLUTION FIXING BY VAPOR 1–5 MINUTES).

Osmic acid shows in *Arbacia* very similar pictures to those obtained by the lipophilic stains. One can see very clearly the basal ring, the tail, the middle piece; the conical part of the head appears darkened and unburnished, which gives us the impression that the substance darkened by osmic acid is located on the surface of the spermatozoön. The substance between spermatozoa is darkened too. On fresh preparations this substance can be distinguished still more clearly. If spermatozoa are shaken in sea-water, and from the clear fluid, which separates in the upper part of the test tube, a drop is taken and submitted

to the action of osmic acid vapor, one sees under the microscope very fine round droplets, black and refractile, evidently originating in the intercellular substance. The preparations by osmic acid give us the impression that there is *on the surface of and between the spermatozoa a substance which contains fat* and which surrounds the cells as a mantle.

5. Sudan III.

Sudan III. shows in the same way as osmic acid, the presence of fatty globules accumulated between the spermatozoa after treating them with the reagents.

6. The Macrochemical Test with Victoria Blue.

If 1 per cent. Victoria blue in distilled water is mixed with ether, one obtains, after shaking, an emulsion which, after 10–30 minutes, separates in two distinct layers: the *non*-stained ether above, and the strong colored Victoria blue below. If to the same mixture one adds dry sperm, after shaking, the ether separates again, but now it carries with it the fatty substances from the spermatozoa. These substances are already stained by Victoria blue, and, therefore, the ether in the upper part of the test tube is blue. If, instead of spermatozoa, we use only the fluid separated after centrifuging a sperm suspension, we may have a complete transfer of color into the ether, the lower layer, representing the water of the Victoria blue solution, being now uncolored. This reaction corroborated by the tests of Schumacher and the reactions with Sudan III. and osmic acid demonstrates that around and between spermatozoa there is a substance rich in unsaturated and neutral fats.

7. Observations on Living Spermatozoa in Sea-water.

Observations are made with the oil immersion lens. By varying the focus, the strength of the light and the eye pieces, one can see quite clearly many of the facts obtained by staining methods. Without doubt, the value of this kind of observation is very great, especially when combined with the above mentioned methods; more and more one reaches the conviction that there are in the spermatozoa two different zones: a darker one at the

periphery, which becomes thicker towards the connecting region between head and tail, where it constructs a large ring. The central zone is more refractile. It is larger towards the connecting region and diminishes towards the apex.[1] Almost all transformations of the ring can be seen in fresh spermatozoa; the sensillæ amœboideæ show most clearly. The migration of substances can be deduced by seeing the changeable sizes of various parts of the *same spermatozoön* during the observation.

In the preceding observations, there is one fact which deserves more emphasis: this is the *stickiness of the pointed part of the head, and of the connecting region too.* Very frequently, and chiefly in some particular reactions, taken into consideration below, the spermatozoa bunch together, sticking by these two regions. By careful and patient observations of fresh spermatozoa, during longer time and in various media, one gets the impression that the spermatozoa eliminate through the point of the head (where really there is an exceedingly minute opening) very small amounts of an extremely sticky substance.

8. The Effects of Egg-water on the Spermatozoa.

The mutual relations of the various constituents of the spermatozoön, being established and knowing the lability of these relations, a large field for studying the topographical changes of the principal substances in various media is opened. I shall limit myself here to one point: *the changes which occur in the spermatozoa in egg-water, which might be expected to resemble changes preliminary to fertilization.*

This is the classical phenomenon studied by Lillie.[2] It is very well known at the present time that there is exerted an agglutinative action upon spermatozoa by the sea-water in which sea-

[1] Mischer (*loc. cit.*) noticed the same thing. Ballowitz in *Untersuch. u. die Struktur d. Sp.* (Fische, Amph. Rept.), p. 249, distinguishes in the head of the spermatozoön two different zones, and he says that in the process of swelling "the internal zone leads." In fact, it alone swells the external substance merely changing its place.

[2] Frank R. Lillie, "Studies of Fertilization. VI. The Mechanism of Fertilization in *Arbacia*," 1914, *Journ. of Exp. Zoöl.*, Vol. 16, p. 523. Frank R. Lillie, "Studies of Fertilization. V. The Behavior of the Spermatozoa of *Nereis* and *Arbacia* with Special Reference to Egg-extractives," *Journ. of Exp. Zoöl.*, Vol. 14, 1913, p. 515.

urchin eggs have been for a short time. The reaction is instantaneous. The spermatozoa exhibit great activity and bunch together, for a while, in compact clusters of various sizes.

What is the immediate cause of the agglutination residing in the spermatozoa? Is it due to a change in the arrangement of the substances, or to an active elimination of the sticky substance observed in fresh suspensions of spermatozoa?

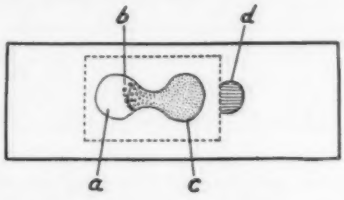


FIG. 10. Arrangement of agglutination experiment; *a*, egg-water. *b*, clusters. *c*, sperm-suspension. *d*, drop of Janus green.

One drop of egg-water and one drop of sperm suspension are placed on a slide (Fig. 10). A very narrow bridge is established between the two drops. Immediately the agglutination takes place where the drops meet, and slowly spreads, so that we have again various stages of a phenomenon on the same slide.

At the left end of the egg-water drop (Fig. 10) the fluid is still clear; at the middle, agglutinated clusters of spermatozoa in great activity; at the right side of the sperm suspension drop, the spermatozoa in various degrees of motion, are in a homogeneous suspension.

In these conditions, one keeps the slides various lengths of time (1–10 minutes). Afterwards one puts very carefully a cover slip with another drop of egg-water underneath (to mitigate the power of capillarity). Many bunches of spermatozoa are spread out, but many other resist, and one can observe, under the oil immersion lens, what happens in the clusters.

A long series of transformations of the individual spermatozoa occur, represented by the next sketch (Fig. 11). These transformations take place under the influence of egg-water only. But in order to see them more clearly, one may add at the margin of the cover slip a drop of 1 per cent. Janus green in

sea-water. The changes shown diagrammatically in Fig. 11 are: (*a*) A normal spermatozoön; (*b*) Initial changes in the arrangement of lipochromatic substance; (*c*) The nucleus swells a little

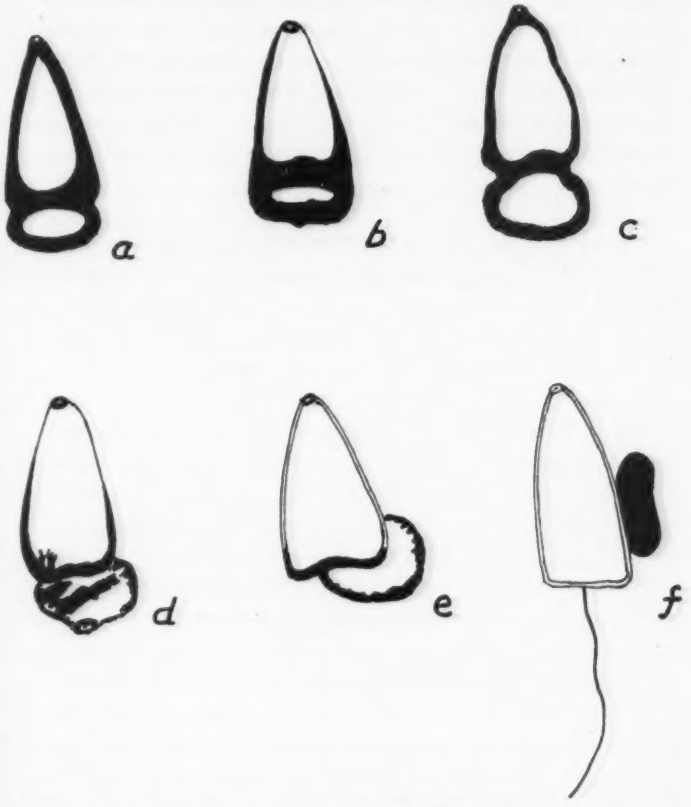


FIG. 11. Behavior of the spermatozoa of *Arbacia* in egg-water; intra-vitam staining with Janus green. See text.

and moves towards the base of the head. At the same time the lipochromatic substance divides into two, a part follows the nucleus, covering it, another part remains in the region of the basal ring. Thus one can see in fresh preparations *on the connecting region* of the spermatozoa two transversal dark zones, between which there is a refractile one. On stained preparations

there are two colored strips and one light band; (*d*) The same process, much more advanced; (*e*) The lipochromatic substance accumulated in greater amount, shifts from the connecting region; (*f*) A round or slightly oval body is thus extruded from the head, and it takes a lateral position, where it persists without any modification, and the whole spermatozoön becomes perfectly quiet. It is to be noticed that the spermatozoa which have extruded this *lateral body* do not swell. On the other hand, the spermatozoa which have not extruded it, swell and become round.

III. DISCUSSION.

Summarizing all the facts revealed by the various methods above described, concerning the topographical distribution of the material in the spermatozoön (in *Nereis* and *Arbacia*) there is to be distinguished a substance stained by lipophilic stains, *the lipochromatic substance;* and another substance very avid for water, *the hydrophilic substance.* The lipochromatic substance is located at the periphery of the spermatozoön, and its thickness is variable in different regions; towards the connecting region it constructs a large ring and the sensillæ; in the tail it takes part in the construction of the tail mantle. This substance is not avid for water, and therefore does not change its volume in various hypotonic solutions. On the other hand, it shifts position readily, and it accumulates normally in the connecting region of the spermatozoön (Fig. 11).

The hydrophilic substance is located in the axis of the spermatozoön, and does not take the lipophilic stains. In the head it includes the nucleus or may be represented exclusively by the nucleus. It is extremely hydrophile, swells in water (hypotonic solutions) and thus enlarges the head of spermatozoön until more than three times diameter. This substance is centrally located in the head and also in the axis of the tail.[1]

Apparently the hydrophilic substance of the head is nothing else than the nucleus and the lipochromatic substance must be

[1] Concerning the sensitiveness of these cells to the changes in the osmotic pressure see: Richard Goldschmidt, "Kleine Beobachtungen und Ideen zur Zellenlehre III Die Bedeutung der atypischen Spermatozoen," *Arch. f. Zellforsch.*, 14 Bd., 1921, S. 290. Richard Goldschmidt, "Versuche zur Spermatogenese in vitro," *Arch. f. Zellforsch.*, 14 Bd., 1921, S. 421. Josef Speck, "Neue Beitrage zum Problem der Plasmastrukturen," *Zeitschr. f. Zellen- u. Gewebelehre*, 1 Bd., 1924, S. 278.

the cytoplasmic component of the spermatozoön loaded with lipoids and lipoproteids.

The cell is surrounded by a very thin membrane, the presence of which is obvious when the heads swell and explode in hypotonic solutions. The membrane must be very elastic and permeable to water. The whole complex is protected externally by a thick *layer of fatty substance*, darkened by osmic acid and detected also by Sudan III. and by macrochemical analysis with Victoria blue.[1] Its chemical composition is still not clearly demonstrated.

Mixing dry sperm with sea-water one can clearly see this substance spreading out on the surface film like oil. In the dry sperm under the microscope, one can see how abundant this intercellular substance is. In this medium the spermatozoa live longer than without it. Presumably the contact of the spermatozoa with the water is very much mitigated by the surrounding fatty medium, so that changes of osmotic pressure influence the spermatozoa gradually, and the modifications of form occur very slowly. This layer of fatty substance may be considered on the whole as a *buffer* between a very sensitive structure (spermatozoön) and a salt solution at a very high concentration (sea-water). Certainly the fatty substance may have some other important significance, too, not yet taken into consideration.

As soon as the concentration of the medium is changed and the fatty medium is modified, the spermatozoa react first by motility, and secondly the mutual relations of the two substances (hydrophilic substance → lipochromatic substance) undergo changes. As a result we have the various pictures of the spermatozoön already demonstrated.

For completion of the general sketch of the spermatozoön there is to be remarked the presence at the distal end (that is

[1] This layer outside the spermatozoön has been frequently noticed recently by various authors: Redenz, E., "Versuch einer biologischen Morphologie des Nebenhodens," *Arch. f. Mikr. Anat. u. Entw. Mech.*, 1924, 103 Bd., S. 391. Gellhorn, E., "Physiologische Untersuchungen an Spermatozoen und Eiern. Ein Beitrag zum Befruchtungsproblem. Sammelreferat," *Arch. f. Mikr. Anat. u. Entw. Mech.*, 1924, 101 Bd., S. 437. Redenz, E., "Versuch einer biologischen Morphologie des Nebenhodes. II. Die Bedeuting elektrolytarmer Losungen für die Bewegung der Spermien," Wilh. Roux' *Arch. f. Entw. mech. d. Org.*, 106 Bd., 1925, S. 290. T. von Lanz, "Über Bau und Funktion des Nebenhodens und seine Abhängigkeit von der Keimdruse," *Zeitschr. f. die ges. Anat. Z. f. Anat. u. Entw. g.*, 80 Bd., Festschr. f. S. Mollier, 1926, S. 177.

to say, the tip of the head) of a minute opening, which it is convenient to call by analogy *micropyle;* and also at the connecting region there is another small ring very easily colored by stains which have affinity for chromatin, especially by *carbol-pyronine.* This is the classical proximal centriole.[1]

We have seen above the various changes of the mutual relations of the two substances in the head of spermatozoön. The end result of these changes is the elimination of the *lateral body.* This elimination phenomenon occurs, also, under various other circumstances, for instance, when spermatozoa are in weak formaldehyde solution, or in some staining solutions, but the process takes place very slowly and quite irregularly. On the other hand, in the egg-water after five minutes almost all spermatozoa show this *lateral body.*

Fresh preparations of spermatozoa of *Arbacia* in egg-water also show quite clearly the elimination of a substance through the pointed apex of the spermatozoön. This appears as a small granule on the points of almost all spermatozoa. The adhesion of the spermatozoa to one another or to other objects is made by means of this granule.[2] In the middle of some clusters one can see groups of such granules very refractile, round and close together. For this observation only fresh preparations are to be trusted, because stains may form very fine precipitates which cannot be distinguished from drops of the eliminated substance.

Very probably, under the influence of some chemical substances included in the egg-water (Lillie's fertilizin?) spermatozoa eliminate through the micropyle a sticky substance, which so long as it still adheres to the apex of spermatozoa keeps them agglutinated. After it is lost from the tip, the spermatozoa spread out again in the fluid. I cannot decide if this substance exists as such in the spermatozoön or if it is produced by a secretory process at the moment of stimulation by egg-water, as in the sense of Bowen.[3]

[1] Here also is the opening observed by Mischer and Ballowitz (*loc. cit.*) and called by the former worker microporus.

[2] Ballowitz, E. (*Zeitschr. f. wiss. Zoöl.*, 1890, Bd. 50, S. 317) called this granule "Spitzenknopf" and noticed its instability (p. 375).

[3] According to R. H. Bowen, who adopts the theory of Nassonov concerning the rôle of Golgi apparatus, "Das Golgische Binnennetz und seine Beziehungen zu der Sekretion. Untersuchungen über einige Amphibiendrusen," *Arch. f. mikr. Anat.*, 97 Bd., 136, the acrosome should be a secretory organ and the granule on

The spermatozoa also undergo in the egg-water more extensive alterations, which are expressed by changes in the arrangement of the substances and as the end result we have the formation of the *lateral body* as described above.

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NESTING HABITS OF SOME ANTHIDIINE BEES.

CLARENCE P. CUSTER AND CHARLES H. HICKS.

The habits of the Anthidiine bees have been studied by Fabre (2), Friese (3), Davidson (1), Melander (7), Newberry (8), Hungerford and Williams (6), Hicks (5), and others. Fabre early considered the genus *Anthidium* to consist of two groups, divided primarily on habits. One he termed "cotton-workers"; the other "resin-workers." The former now comprise the genus *Anthidium;* the latter *Dianthidium*. The habits of these bees have not been studied so thoroughly in America as in Europe. Studies of *Anthidium porteræ* Ckll. have been made by Hungerford and Williams (6) and Hicks (5), and *Dianthidium sayi* Ckll. by Hicks (5).

The writers are indebted to Dr. T. D. A. Cockerell and to Dr. Francis Ramaley for helpful suggestions and for the determination of specimens; to Dr. Edna Johnson for the determination of some plants; and to Mr. S. A. Rohwer for determining some wasps.

We continued a study of these bees during the summer of 1926. The following is an account of our work.

Anthidium porteræ Cockerell syn. *maculifrons* of authors, not Smith.

Hungerford and Williams (6) state that two females of this bee were observed nesting in a bank of coarse, quartz sand near a colony of bembecid wasps. The down of the cells of the nest was thought to be derived from thistle (*Cirsium*) stems or from the inflorescence. The tunnel of one was 10 inches long.

Most of our study of this species has been done at Point East [1] and at Base Line Lake,[2] near Boulder, Colorado. The nests have been found both on hillsides and on level ground, usually in bare areas.

[1] A small hill rising above the plains, 3 miles east of Boulder.

[2] Six miles east of Boulder on the plains.

We attempted to observe whether or not *A. porteræ* digs her own nest or uses one already constructed by another insect. Individual females have been observed as long as 15 minutes at a time entering, spending a few seconds within, and then leaving various types of holes. At other times the bee merely looks in and leaves. We have never seen this or any other species of *Anthidium* digging in the ground.

Sharp (9) states that the species of *Anthidium* do not form burrows for themselves, but use suitable cavities formed by other insects in wood, deserted nests of other bees or empty snail shells.

As far as can be ascertained, no one has before observed an *Anthidium* bee to drive away another insect and take the nest, although it has been generally assumed that it uses the deserted nests of another insect, or a convenient cavity.

While observing a wasp, *Odynerus dorsalis* Fabr.,[1] building and provisioning a nest, *A. porteræ* was seen to drive away this wasp and appropriate the nest for her own. A few days previous to August 2, a female of this wasp[2] had been observed working and nesting. During the morning of August 2, I had been watching this wasp excavating and carrying out moist pellets of earth. Shortly after noon she had apparently finished the nest. The following notes were taken in the field near Base Line Lake between August 2 and 5, 1926 (h).[3]

2 : 50′. See *Odynerus* returning, flying and carrying large lepidopterous larva. She enters hole with it, staying but a minute within, then leaves.

3 : 17′ 05″. Back without larva. Flies in wide circles after which alights near hole, tapping edge with antennæ a few times. Enters. Backs out. Flies away. The hole is 5 mm. in diameter.

3 : 38′ 10″. Back with another larva which she takes within. Out, and flies away.

3 : 40′ 15″. *A. porteræ* is seen flying low along ground and entering holes nearby.

[1] Kindly determined by Mr. S. A. Rohwer.

[2] An account of the habits of this wasp given by C. H. Turner, in BIOL. BULL. 42, 1922, p. 153–172, 6 figs.

[3] Initial (h) refers to notes of Hicks; (c) to those of Custer.

3 : 41′ 08″. She comes to hole of wasp nest. Enters and remains within.

3 : 43′ 33″. Bee still within. Wasp returns, alighting at edge. *A. porteræ* is near top of hole with jaws spread in a threatening manner, and head blocking the entrance. Wasp jumps at bee in hole, then flies away. Back and strikes at abdomen of bee as the bee backs out of hole. Bee and wasp leave, fighting. Wasp soon returns, alights near hole, tapping it with her antennæ. Enters and is pulling out one larva as *A. porteræ* returns. Bee strikes and tries to bite wasp with her mandibles, a number of times. The wasp flies with larva and drops it 10 inches from hole after which she flies away. Bee then goes inside. Wasp returns but pays no attention to larva which she dropped but comes to hole and looks in for a time. She then flies away. *A. porteræ* leaves.

3 : 54′ 01″. Bee enters hole.

3 : 56′ 52″. Goes away.

4 : 05′ 50″. Bee returns.

4 : 06′. Wasp returns. Tries to enter, jumps back and flies away.

4 : 07′ 02″. Bee flies away.

4 : 18′ 15″. *A. porteræ* enters. Believe carried something inside.

4 : 18′ 40″. Leaves.

4 : 20′ 55″. Bee returns, carrying a load of down.

4 : 21′ 05″. Flies away.

4 : 23′ 40″. Returns with load of down.

4 : 24′ 15″. Leaves.

4 : 26′ 29″. Returns with down.

4 : 27′. Leaves tunnel.

4 : 27′ 01″. Wasp returns. Looks in. Goes in and comes out almost at once. Flies a few inches away. Returns. Looks in. Goes in. Out and is ready to go in again when *A. porteræ* returns with load of down. She strikes wasp and both fly. The bee follows wasp fighting and striking her while in the air. The wasp circles back to the nest followed by bee, and tries to go into the hole. The bee repeatedly strikes wasp as latter tries to enter. *A. porteræ* drives her away, following her out of my view.

4 : 29′ 33″. Neither have come back.

4 : 31′. Wasp back. Looks in and flies away. Back again soon. Looks in. Goes in. Flies away in a circle. Back to nest. In. Out with some down in mandibles. In again and out with down in mandibles. Six more trips in and out with down. Then out dragging larva by head. Gets larva half out and puts back in at once as though she sees mistake. In and out with five more loads of down. Is now 4 : 35′. Comes out again with load of down at which time bee returns flying at wasp and hitting her a number of times. Bee then enters hole. Wasp is back very soon again. Enters hole in which bee is in but backs out at once. Follows an active four minutes in which the wasp repeatedly flies about hole, returns to it, looks in, flies away a few inches, back, etc. The bee is well up the tunnel blocking the entrance, during this period. The wasp flies away.

4 : 41′. *A. porteræ* is still in the hole but has gone down so that cannot see her.

4 : 42′ 55″. Bee out. Flies around and back into tunnel. Goes down into cell.

4 : 46′ 10″. Wasp flying rather high over nest but does not stop.

4 : 48′ 20″. *A. porteræ* out. Flies a few feet away and back carrying nothing.

4 : 53′ 20″. Repeats above.

4 : 55′ 20″. Repeats.

4 : 57′. Bee backs out dragging larva carried in by wasp to the outside. Drops 2 inches from nest. Enters. Out head first. Leaves.

5 : 01′ 02″. Back with down.

From this time until 6 : 30 the bee carried 17 loads of down. The wasp was not seen again. The next day the bee carried 18 loads of down. The day was partly cloudy. On August 4, by 10 o'clock the bee was collecting pollen and by 9 : 15 A.M., August 5, the nest was complete with the tunnel filled with pebbles to the surface of the earth (h).

Anthidium bees have been observed by many students to use down from various plants growing in the vicinity of the nests. Fabre (2) found that some would even readily make use of that

from introduced plants. He states that *A. diadema* will take the cotton for its work from any suitable plant growing near its nest. This bee nests in hollow reeds placing as many as ten cells in one stem. The most we have found here have been two in one nest, more usually one. This is due to the fact that most of ours have probably taken over wasp nests providing space for but one or two cells.

According to Friese (3) certain European *Anthidia* obtain their down from the leaves of different hairy plants, among which are especially such plants as *Stachys*, *Ballota*, *Cydonia*, *Verbascum*, *Populus* and *Gnaphalium*.

A. porteræ gathers down from the underside of the leaf of *Cryptanthe gracilis* Osterh.,[1] by scraping it up towards the apex with her mandibles.

After visiting several leaves she then holds the ball gathered with the mandibles between the thorax and front legs and flies with it thus to the nest. A given individual usually returns to the same group of plants to obtain the down. One bee averages two and one-half minutes in securing a load (c).

We have observed a bee to go a distance of over 50 yards in securing the down although some others have been seen to go but 5 yards away while one at White Rocks went but 3 feet from the nest.

In August, 1926, *A. porteræ* was observed to gather down from the stems of live *Artemisia canadensis* Michx.[1] One female was observed while she went from plant to plant visiting a large number on one collecting trip. This bee scraped the down in much the same manner as those observed working on *Cryptanthe*. It would seem that this latter plant is much better for the bee as it has more down and requires less work to obtain it (h).

On July 20 and 21, 1926, notes were taken on the provisioning of a cell, an extraction of which follows (h).

One nest was watched continuously for a number of hours. The opening to the tunnel was slightly enlarged so that the bee could be seen working within. She would return to the nest carrying the down with her mandibles, enter, and arrange it

[1] Kindly identified by Dr. Edna Johnson.

into a cell shaped for the reception of the pollen. She usually turned herself around once and sometimes two or even three times in the placing and working of each load.

In returning to the nest the bee came direct, leaving in the same manner. Although the weather was partially cloudy the bee carried 26 loads of down in two days, an average of six minutes and thirty-eight seconds for each load.

The time of individual trips varied from 4′ 49″ to 16′ 05″. The time spent within the nest, arranging the down varied from 1′ 10″ to 2′ 20″. The time taken to get the down is determined by weather conditions, distance secured from nest, time of day, etc.

At the time of provisioning, the cell had an inner cavity, a little larger than the bee. She began at 1 : 40 to carry pollen and between this time and 3 : 30 carried 6 loads. The time spent within the nest averaged about 2 minutes. On arriving, the bee opened the top of the cell, entered head first, came out, and backed in, forcing the abdomen inside. While here, she removed pollen from the ventral scopa and deposited it in the bottom of the cell. Before leaving the nest, she always partially closed the cell by raking the down together at the orifice. This may make it more difficult for parasites to find or enter the nest.

After depositing the pollen, the bee was not seen to enter the cell again until she had returned from another collecting trip. If nectar was deposited after each collecting trip, it of necessity was deposited first when the bee had her head within. However, the first pollen was seen to be dry and it was not learned if separate trips were made to gather nectar or not. The final food for the young is very moist (h).

After provisioning, laying the egg and closing the cell the bee fills up the tunnel, usually with pebbles. These may be selected from a small area over 9 yards from the nest, in rare instances, but more usually from 1 to 3 yards away. A number of times the bee has availed herself of the pebbles of a large red ant nest (*Pogonomyrmex occidentalis* Cresson).[1] The number of pebbles used is at least enough to fill the tunnel above the cell even with the surface of the ground. The size of the tunnel and the

[1] Kindly determined by Professor T. D. A. Cockerell.

distance above the cell varies. From 135 to 300 pebbles are used, 250 being about the average.

Sometimes a bee loses its hold or attempts to carry a pebble too heavy and is forced to drop it. She then goes at once to the place, from which she secured the one dropped, for another. However in one instance, upon dropping the pebble midway to the nest, the bee alighted at the spot, seized another, and continued on her way. The bee usually drops the pebble from above the hole into the tunnel but on a few occasions she was observed to alight on the edge before dropping it in. Sometimes the pebble fails to hit the hole and remains close to the edge. While filling the hole the bee frequently enters to arrange the pebbles. In doing this she turns them with her mandibles and fits them into position snugly. In only one instance, after the bee had gone inside to arrange the pebbles, have we observed her on leaving to rake in some pebbles and soil from near the edge with her mandibles and fore legs.

On July 24, 1926, found bee filling tunnel. She was watched until the end of this period. The following is an extract from more detailed notes taken in the field (h).

At 8:50 A.M. found *A. porteræ* entering hole in ground with piece of dry dirt. The hole was large enough to see inside and it was noted that only a few pieces had been deposited for the down was not covered. When first observed the bee was carrying material from a place 12 feet east of nest. She carried 10 loads in an average time of 12 seconds. Each time the bee alighted on the ground and selected the piece of dirt. These chunks varied somewhat although the average was quite uniform.

After she carried a few more, a circle of stones was placed about the nest while she was after a load. The stones were about the size of a hen's egg and were 3 inches from hole. On returning she circled 3 times about the hole, then flew 10 feet away, returned, alighted on ground, returned and entered hole. She then went to collect again and continued, returning direct and without further hesitation, paying no more attention to the change. After 15 more loads she changed her place of selecting materials to 18 feet south of nest. The bee, after every few loads, went and arranged them. After 56th trip changed back to position

12 feet east. After 113th trip (9:47) bee went to feed on *Psoralea* plant. She was followed and was found to visit 83 flowers in 6 minutes. She then carried 3 loads into nest and fed again, visiting 15 more. The 139th trip ended at 10 o'clock.

The bee had no sooner alighted, after the 155th trip than a male met her. The period of mating lasted 25 seconds after which the female went on with her work as before. In one instance she carried in a piece of old stem which was so long that it was bent back to get into the hole.

She made in all 240 trips between 8:50 and 10:26 at which time the tunnel was filled even with the surface. This bee carried in almost entirely pieces of dried mud; all others observed have used mainly pebbles. The nest was dug up and consisted of two cells. This nest was of the nature of those made by *Odynerus dorsalis* Fabr. The lower cell was opened and contained a small larva of *A. porteræ* which later died. The upper was not opened until in September at which time a cocoon had been formed containing a live, mature larva. In this instance one mating took place after the egg of the two cells had been laid, both eggs hatching. It is probable that *A. porteræ* mates more than once. Other matings have been observed to take place while the female was collecting pollen for the cell. Once a female of this species was found to be mating with a male, *A. porteræ amabile* Ckll., a variety with more red on the abdomen (h).

The mass of pollen, semi-fluid in consistency, of the cell of *A. porteræ* is 10 mm. long, flat on the top surface and curved on the base. The egg lies flat on top of the mass of provision. A larva 2½ mm. in length was found to be partially embedded in the food. The exact larval period has not been ascertained although a number of cocoons have been formed in the laboratory. The larvæ of those opened never developed much further and it was thought that this might be due to the pollen drying out as well as to possible mechanical injury (h).

The size of the entire cell including the down varies a little, due to the size of hole in which the down is placed. One which was about average in size measured 17 mm. in length, being 12 mm. wide at the bottom and 10 mm. wide at the top. Pellets

of excrement mixed with down were all about the down and between the cocoon and the outside (h).

The outer surface of the cocoon has a thin layer of rather light, loosely woven silk. Removing this the denser, darker cocoon is found. An average cocoon measured 10 mm. in length and 5 mm. wide, being oval in shape with the ends somewhat flattened. The anterior end bears a mammillary point which externally contains an opening. This point is not so long nor so conspicuous as the one on the cocoon of *Dianthidium sayi*. One large cocoon measured 12 mm. in length and 7 mm. in width. The cocoons found have been reddish brown. One of the cocoons described, held to a bright light showed the outline of the larva within (h).

We made certain investigations in which we attempted to find if *A. porteræ* would carry pebbles indefinitely to the nest. While the bee was away for a few seconds to obtain another pebble we removed the one, with a pair of forceps, she had last dropped in. Thus the nest was kept continuously empty or partially so.

On July 27, 1926, at Point East at 3:15 P.M. we found a bee carrying large pebbles from the nest of the red ant (*Pogonomyrmex occidentalis* Cress.) 9 yards away. The nest at that time was about half full. We removed 175 pebbles by 5:20 P.M. when the bee quit working for the day. The nest was watched until 7:00 P.M. and from 7:15 A.M. the following morning but the bee was not there at either time and no pebbles had been carried in the interval. She started again to carry pebbles at 7:55 (July 28). From 7:55 to 8:20 she carried 100 stones, which is an average of 4 stones a minute. Later in the day (10:00 A.M.) she was found to be averaging 8 a minute, probably due to the increased intensity and heat of the sunlight. She carried 311 pebbles before noon, having fed early in the morning, suddenly stopped and was seen no more. Although the nest was watched for days, no more pebbles were carried. The total number carried was 741. The bee was not frightened away and apparently quit voluntarily, leaving a number of pebbles in the nest.

To another nest a bee carried at least 400 pebbles and suddenly left, although the hole was kept partly empty as in the other case. Before stopping, in each instance, there was no apparent slowing

down of the work, nor cloudy or cool weather. The bee stopped during weather conditions very favorable for work.

In two instances, I have found the nests of some species of *Anthidium* in which the tunnel was unusually short, and over and above which she had carried a mound of pebbles instead of just filling even with the surface, as in the case of nests having longer tunnels. In one instance 135 pebbles and in the other 75 had been carried, more than enough to fill the tunnel (h).

From our investigations it would seem that *A. porteræ* will not carry pebbles indefinitely even under ideal weather conditions.

Whenever the cell becomes damp and mold develops, as sometimes after rain, the nest is deserted.

Both males and females have been found resting in the dry pods of yucca stems during rainy or cloudy weather, in cavities in decayed wood or resting, perched bird-fashion on stems during the night.

During the day when the weather is fine the males dart from flower to flower and are often seen hovering humming-bird fashion in the air. Part of the day is spent in feeding and part in searching for the female. He often darts upon the female while she is working, copulation taking place on the ground. The duration of this period is about 30 seconds. As yet we have reared no parasites.

Summary.

1. No females of *Anthidium porteræ* have ever been found digging a nest.

2. On one occasion she drove away a nesting wasp, *Odynerus dorsalis*, and used the nest for her own.

3. The nests found in which the cells of the bee are placed have in general been such as are made by wasps or other insects nesting at the time.

4. *Anthidium porteræ* is solitary and is not found nesting in close association as *Dianthidium sayi* and others.

5. The cell is constructed of down and the tunnel above filled to the surface of the ground, usually with pebbles.

6. When the stones are removed artificially from the tunnel, *Anthidium porteræ* probably will not replace them indefinitely even under ideal weather conditions.

Dianthidium sayi Cockerell syn. *interruptum* Say. (nom. preocc.).

The bees of the genus *Dianthidium* have been known to construct nests of resin on rocks, in stems of plants, in deserted snail shells and other places, but, so far as we know, seldom in the ground as does *Dianthidium sayi*. We have found no reference to the nesting habits of this species except that in 1926 by Hicks (5). This latter information was derived from a number of cells dug up from a small area at White Rocks, during fall of 1925 near Boulder, Colorado. We have obtained our field notes during the summer of 1926 mainly at this locality where two colonies, one hundred yards apart, were found and studied.

The six colonies of *D. sayi*, observed during the latter part of the summers of 1925 and 1926, were all located on hillsides that face south and slightly east. Scattered over these hillsides were tufts of *Bromus brizæformis* Fisch. and Mey.,[1] to the roots of which some of the resin cells were attached. We find that the species may nest as a colony of from eight to fifty or more females, although one apparently solitary female was found nesting in a vertical clay bank. The colony is at its maximum activity during the month of September.

The picture shows a small colony of *D. sayi* on a grass-covered mound of sandy soil one yard in diameter. Base rock surrounds this on all sides, the nearest soil being five feet away. Tall

[1] Kindly determined by Dr. Francis Ramaley.

stems of *Bouteloua oligostachya* (Nutt.) Torr. and *Bromus brizæformis* Fisch. and Mey., rise a foot or so above the colony; thus chaff, small sticks, etc., are abundant. The nests were marked, as seen in the picture, 1 to 16 by small numbered strips of paper securely weighted with sand. Nest 7 has been plugged up. The picture shows bee *H*, whose thorax is white for identification, very near and to the left of this nest. She is standing on her hind legs with the fore legs on the bank just above the nest. Nest 4, hidden from view, is a foot behind 2 while nest 14 is a quarter inch above 5. Nests 11, 12, 13, 15 and 16 are hidden from view in the grass to the left of 14. Nests 7 and 8 are about twenty-five inches apart (c).

All bees concerning which notes were taken were marked with waterproof white paint. Thus the thorax of the bee seen beside label 7 is white. Bee *C*, on whose habits many important notes were taken, was marked as follows: (1) Left half of dorsal surface of last abdominal segment white; (2) A black area present on left side of fourth dorsal abdominal plate completely surrounded by a yellow tegumentary band which area, on right side of same abdominal segment, was not completely surrounded; (3) Right postero-lateral quarter of mesothorax dusty, probably due to some resin sticking there at one time and dust adhering to this (c).

One bee was seen to have two nests in the process of construction at which she worked more or less alternately. Later, five such bees were observed. These were shown to and verified by Hicks. The following short extract illustrates this habit in the case of one bee: Bee *C* is constructing two nests which are nine inches apart. Into one nest she carries pebbles, lumps of dirt, chaff and such sticks as she can handle with her mandibles. Then she flies directly to the other nest and does the same thing there. Thus she carries the following number of these articles

To Nest 1:	Then to Nest 2:
6	6
4	5
9	0
(Then a male arrives and they mate while she has a stick in her mandibles (60 seconds). He leaves and she continues her work after carrying this stick to nest 1.)	
23	0
(She flies to flowers of *Grindelia* several yards away, and feeds there for ten minutes; then she returns to work as before.)	
5	1
5	0
Here she flies to the fields. One of the sticks is one and one half inches long (c).	

The following account shows this habit in another individual: Bee *D* goes into nest 3 for fifty-five seconds. After going out and in several times, she leaves, flies slowly over to nest 4, nine inches away, and goes in at once. Presently she leaves this nest and flies to some nearby flowers to feed. In one minute she arrives at nest 3, leaves after a few seconds, and flies slowly over to nest 4 which she enters. After one half minute, she leaves, feeds for five minutes and goes into nest 3. She comes out, three quarters of a minute later, goes into 4 and spends one half minute there before leaving for the fields to feed (c).

It has not been observed whether or not *D. sayi* digs the hole for its nest and so it can not be proved that one bee has attended to two holes from the first. However, the proximity of the nests to one another indicates that it does the former. It might easily be that each summer, when the colony began to nest, there was but one bee to each hole. Then when some of the builders died, before completing their nests, others of the colony would appropriate these deserted nests and finish them along with their own. The following examples are taken from the notes to give evidence that temporarily deserted nests of *D. sayi* are readily taken over by other members of the colony:

Example 1.

Sept. 14, 1926. While bee *I*, the owner of nest 8, is in the field, another member of the colony comes and goes into the nest. Thirty-five seconds later, bee *I* arrives and goes into

nest 8 also. Presently the owner backs out and is followed closely by the intruder. The former alights on the intruder's thorax, biting with the mandibles at the junction of head and thorax. A short, lively tussle follows, after which the intruder escapes and the owner goes on working.

Example 2.

Sept. 17, 1926. Bee *D*, which has been constructing nests 3 and 4, has now spent ten minutes carrying chaff, etc., into 4. In the meantime, bee *I*, which has been working on nest 8, arrives from one of her numerous trips to the field and goes into nest 3. She at first makes regular trips carrying soil out of this nest. Then she seems to be carrying pollen in. The owner of nest 8 has been working on 3 all day and has but rarely visited 8. The owner of nests 3 and 4 has continued to carry chaff, etc., solely into 4, eventually plugging it up. The next day the owner of nest 3 returns. The owner of nest 8 sees her return and so is now working as before on 8 (c).

Five bees by this time (Sept. 17) have been seen repeatedly to visit two nests apiece. To summarize, they are: (1) Bee *C* to nests 1 and 2; (2) Bee *D* to nests 3 and 4; (3) Bee *E* to nests 5 and 14. (4) Bee *I* to nests 8 and 3 (to 3 only to-day); (5) Bee *H* to nests 6 and 7. There are eight bees working in the colony. Of the sixteen nests, nine are open and seven are plugged, but of the latter, numbers 7, 15 and 16 are not being worked upon by the bees. Thus the eight bees of the colony are working on thirteen nests, and three of these bees are seen to work on but one nest apiece (c).

On September 26, we dug up the colony and found 151 completed cells from the 16 nests, which is an average of 9.44 cells to a nest. This was practically the end of the nesting season, for at this time and later the colonies of *D. sayi* were deserted.

It is interesting to note, from a standpoint of comparative entomology, that a wasp, *Sphex varipes* (Cress.)[1] has been observed to be taking care of two nests also (h).

From these facts and others in our study of bees, we have found that there is sometimes considerable variation in the habits of different individuals of a given species.

[1] Kindly determined by Mr. S. A. Rohwer.

The picture shows bee *H* in one of her numerous visits to the plugged hole 7. Her action suggests that a bee retains the memory of her nest for at least a week after it has been completely plugged up and that her visits are made to it to insure its security from enemies. A brief extraction from my field notes illustrates her action: "Bee *H* goes into nest 6 for one and one half minutes, comes out and goes over to plugged hole 7. She spends about ten seconds here. Upon arriving at 7, she first puts her head down against the plug, rubs it with face and mandibles and scratches weakly with fore legs for three or four seconds. She then stands erect before the nest on her hind legs with her fore legs against the bank above the plugged hole (see picture). After standing thus motionless for about five seconds, she flies away to the fields. She repeats this procedure many times daily in like manner. Only occasionally does she first go to nest 7 and then to 6" (c).

While bringing out soil from within the nest, after the tunnel has been constructed, bee *D* has been observed to back out, raking it with her forelegs. She usually leaves this within an inch of the entrance although in a few instances she took it out in this manner as far as six inches, and once ten inches (c).

D. sayi obtains the resin used in the construction of the nest from the small sunflower, *Helianthus petiolaris*. She obtains this resin, with her mandibles, from the stem and partially or totally dried leaves which have small droplets and plates of it distributed over them (c).

Fabre (2) studied "resin workers" for many years but states that he never saw a bee get resin. Friese (3) states, in reference to the source of resin used by a species found in Europe, that it is probably obtained from the buds of the pine. Hacker (4) inferred that *Megachile rhodura* used resin for its nest since he observed members of both sexes visiting a *Eucalyptus* tree, from which resin had oozed, and rasping the resin with their mandibles. However, he did not find *M. rhodura* nesting. Melander (7) presumes that the resin used by *Anthidium texanum* Cresson was obtained from cedar. Hungerford and Williams (6) say, in their account of *Dianthidium concinnum* (?) Cresson: "The nest of this insect is composed of pebbles glued together

with resinous cement which may be derived from the stems of *Helianthus* which are often infested with a small lepidopterous borer that causes an exudation which usually attracts a host of Hymenoptera."

The following field notes were taken, giving the resin activities of bee *C* during one hour:

12:57½. Arrives with resin in mandibles at nest 2. Kneads resin with mandibles.

1:00. Obtains a piece of chaff an inch away from nest and inserts it into the resin which by now she has shaped into a plug over the ends of the pieces of chaff projecting from the burrow.

1:06¾. Carries piece of chaff to nest 2.

1:07. Obtains small stick and flies with it to nest 1.

1:07½. Flies away. I follow her. She goes to some flowers of *Grindelia* and feeds.

1:09½. Flies to some dried plants of *Helianthus petiolaris*. Picks a plant without many red and black ants present and scrapes resin off with mandibles. She visits three plants.

1:12¾. Arrives from fields at nest 2. Has in her mandibles a load of resin about one fourth as large as head. Distributes resin over ends of chaff projecting from the entrance.

1:21. Flies to nest 1.

1:21½. Leaves nest 1. Goes directly to sunflower plants and secures resin.

1:24. Arrives at nest 2.

1:25½. Leaves nest 2.

1:30. Arrives and leaves after two seconds. I follow her to several flowers of *Grindelia* where she feeds.

1:32. Arrives at nest 2 and leaves, after two seconds, for the fields.

1:34½. Arrives at nest 1 with white resin in mandibles. Works here with the resin in the construction of a plug.

1:36¾. Obtains large pebble two inches away and inserts it into resin of nest 1.

1:38. Flies to nest 2 and works with plug there.

1:42. Carries stick to plug at nest 2.

1:42½. Leaves nest 2 and feeds at flowers of *Grindelia* and of *Helianthus petiolaris*.

1:46. Arrives at nest 2. Leaves after two seconds and again feeds.

1:48. Arrives at nest 2. Walks all over colony looking into several nests and returns to this one.

1:49. Flies away.

1:51. Arrives with resin at nest 2.

1:51½. Obtains piece of chaff and takes to nest 1. Gets stick midway between nests 1 and 2 and carries it to nest 2.

2:01. Leaves nest 2 and obtains resin after feeding on flowers of *Grindelia*, etc. (c).

The entrance to the tunnel, after the completion of the cells, is closed by a plug of resin, chaff and other materials. From several plugs taken out, we find that the resinous part is about 2 mm. thick. Usually, in constructing the plug, the bee first places a few pieces of chaff, sticks, etc., in the entrance and then builds a resin cap over the projecting ends. Beyond the plug, towards the cells, the tunnel is often empty and may be coated with resin.

One of us (Hicks) (5) has described the cells of *D. sayi*. Last summer it was not known whether all the materials found in the resin had been carried there by the bee or were there by chance. Observations this summer show that she obtains the chaff, sticks, etc., which are found mixed with this resin, from the vicinity of the nest. In some instances she has been found to carry fifty or more pieces of chaff, etc., into one tunnel until it was filled. After this she finished the nest with a resin plug. The resin cells are often found attached to the grass roots in similar fashion to peanuts on the stem.

D. sayi varies considerably in the distance which she carries the stones, dried mud, etc., with which she constructs the nest. Bee *J* (attending to nest 9) is seen to make many trips with plates of dried mud from an old mud puddle, six yards away, to her nest. Bee *E* (attending to nests 5 and 14) is seen to carry many large pebbles to her nest from a sand pile eleven yards away. She places these in nest 14 after having plugged up 5 (c).

D. sayi may also obtain chaff and sticks from a distance varying from three inches to three yards away. One individual selected material near the nest but did not repeatedly obtain it from the

same place; another repeatedly visited a more distant source. This latter was usually found to reject several pieces of chaff, etc., before taking one to the nest (h).

The female of this species (bee *C*) was seen to mate twice within three days. The first mating, on September 13, is recorded in field-notes given on page 270. The second mating is given in the following extraction: On September 16, bee *C* leaves nest 1, which is now almost totally plugged up with chaff, etc., and flies to some flowers of *Grindelia* to feed. She then flies to some small sunflowers nearby and feeds there also. Finally she goes to a flower of *Lygodesmia juncea*. While feeding there, a male arrives and they mate. This mating takes sixty-five seconds. The female is grasping the stamens of the flower with the fore and mid legs and is touching the hind legs repeatedly on those of the male. This is apparently the first record of a wild bee mating more than once (c).

The male has been observed to await at the colony the return of a female. As soon as she arrives, he darts at her to effect copulation and in some cases they collide in mid-air, both falling to the ground. The female, with partially outstretched wings, rests motionless except for a slight movement of the hind legs on those of the male. The male has the female grasped around the third abdominal segment with the forelegs and beneath the abdomen with mid and hind legs while the tips of his antennæ are bent forwards and outwards. His only movement is a relaxation and contraction of the abdominal segments and a slight waving of the tips of the antennæ. The average time for copulation, from seven examples, is very close to a minute (59.7 sec.). We have observed that mating may occur both at the colony and at the flower while the female is feeding. Although the male is absent from the colony most of the time, nevertheless the majority of the matings observed have taken place at the nest.

Mating probably does not interfere with the nest-building activities as evidenced by the field notes on the first mating of bee *C*. Furthermore, a female has been seen, returning laden with pollen, to mate, after which she immediately went into the cell, deposited the pollen and went on with her work.

D. sayi has been observed to feed on the flowers of the following plants:

(1) *Grindelia squarrosa.*
(2) *Helianthus petiolaris.*[1]
(3) *Sideranthus spinulosus.*[1]
(4) *Lygodesmia juncea.*[1]

After a rain, which partially filled the entrances of some holes, we completed the closing of these with wet sand to observe the later action of the owners. One, entrapped within the nest, used the mandibles to remove the plug; another, returning from the fields, also used her mandibles to gain entrance. The latter was observed to place head and mandibles into a small excavation, which she had formed, and to walk in a clockwise direction around the hole with the tip of the abdomen describing a circle. The forelegs were practically useless in this process. The abdomen was not used in this case to pack the soil in repairing the walls, as has been observed in a bee of the genus *Augochlora* under similar circumstances.

D. sayi has not been found feeding beyond a radius of one hundred yards from the nest. However, of six marked females released four hundred and fifty-five yards from the colony, four returned within five hours. The shortest time required to return this distance was twenty-one minutes. Two specimens released one hundred yards from the nest returned shortly, the first within fifteen minutes, after feeding along the way (c).

The only parasite of *D. sayi* which has been bred is a fly of the genus *Villa* (Anthrax),[1] Hicks (5). Some mutillids and chrysids may be parasitic since they have been frequently observed to enter the open or partially plugged-up nests.

Summary.

1. Several instances of a female bee, *Dianthidium sayi*, working alternately on two nests in the process of construction, have been found. This has also been observed in a species of wasp, *Sphex varipes*. Apparently these are the first records of this habit.

[1] Determined by Professor T. D. A. Cockerell.

2. Resin, used in the construction of the nest, is obtained from the leaves and stem of the small sunflower, *Helianthus petiolaris*.

3. *D. sayi* is one of those wild bees whose members nest in close association with one another.

4. A female of this species was seen to mate more than once.

5. *D. sayi* seems to be nearly free from parasitism.

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ORIGIN OF THE MESODERM AND BEHAVIOR OF THE NUCLEOLUS IN REGENERATION IN *LUMBRICULUS*.[1]

LEONARD P. SAYLES.

INTRODUCTION.

The microdrilous annelids have been the center of considerable interest on the part of students of regeneration particularly from the point of view of the origin of the cells which form the new tissue. While the production of new endodermal and ectodermal structures from cells of the corresponding old parts is generally accepted, the origin of the mesoderm in the bud has been a point of contention. Hepke ('97) in Naids, von Wagner ('00 and '06) in *Lumbriculus* and Abel ('02) in *Tubifex* and *Nais*, among others, are of the opinion that the new mesodermal structures both in anterior and in posterior regeneration are of ectodermal origin. Randolph ('92) in posterior regeneration in *Lumbriculus* reports their formation from comparatively unmodified cells of the old mesoderm to which she gives the name "neoblasts." Iwanow ('03) in *Lumbriculus* and Krecker ('10) in *Tubifex* and *Limnodrilus* verify her conclusions. They find that in anterior regeneration, however, cells from old specialized mesodermal structures form the new portion. One of the features in the descriptions of the cells of the bud region by these workers is the prevalence of large nucleoli in the cells involved in the regenerative activity. Krecker ('23) gives a rather complete description of the origin and migration of the neoblasts in posterior regeneration. Studies on the origin of the new mesoderm in regeneration together with observations on nucleolar changes in these and other tissues are reported in this paper. For invaluable advice on this work I am indebted to Dr. J. W. Wilson of Brown University at whose suggestion the problem was first undertaken.

[1] This paper forms a part of a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Brown University, June, 1926.

Materials and Methods.

The species used in this work was identified for me by Professor Frank Smith, of the University of Illinois, as of the genus *Lumbriculus*, and probably the species *inconstans* Smith. Anæsthetization was avoided as far as possible. When it was necessary, a one eighth of one per cent. solution of chloretone or a four per cent. solution of alcohol, redistilled in glass, gave equally satisfactory results. For histological fixation Zenker's fluid or Bouin's Picroformol were used; best results were obtained with the latter. It was not necessary to anæsthetize the worms first. An individual to be fixed was placed on a glass plate and straightened against a glass slide. Another slide was then pushed up until it came in contact with the worm, care being taken not to crush it between the slides. In this process only a very small amount of water was used. The fixing fluid was then slowly added and its surface tension, as it was drawn underneath the two slides, held the worm firmly in place during the preliminary fixation. This method is a modification of one described by Welch ('13). As a stain Heidenhain's iron alum-hematoxylin method was used for the most part, though preparations were also made with Delafield's hematoxylin and eosin, used both as a direct and as a regressive stain. On carefully controlled regressive staining the chromatin granules were stained with the hematoxylin while the nucleoli took the eosin, indicating that they were true nucleoli or plasmosomes.

Nuclei and Nucleoli in the Cells of Uninjured Individuals.

As this paper is to deal at some length with the cytological changes, particularly nuclear and nucleolar, which occur during regeneration, it is perhaps advisable first of all to discuss more or less completely the conditions which exist in uninjured individuals. Under this heading the usual appearance of cells in the hypodermis, setigerous glands and intestine, including those differences which may exist at the growing region of the tail, will be described.

Hypodermis.

In all the epithelial cells of the hypodermis the nuclei are quite large, all gradations from an oval type (the average measurement of twenty-five of which is 3.3 x 5.8 μ), to the practically spherical one (averaging about 4.2 μ) are to be found. In each a very minute nucleolus, appearing as a mere dot under a magnification of 1,000 $\times$, is visible (Fig. 1). Cells of this type form all of the hypodermis from the second segment to the growing region of the tail. At this end, just anterior to the place where the basement membrane is discontinuous due to the formation of the ventral nerve cord, the hypodermal cells are slightly elongated. The nucleoli are slightly larger than in the cells located more anteriorly (Fig. 2). Cells of this type are probably intermediate stages between the active ones of the growing region and the typical hypodermal cells found in the older part of the body. Such an elongation of the cells becomes even more marked where the nerve cord is forming with the result that the hypodermis in the growing tip is two or three times as thick as in the rest of the body wall. This elongation of the cells is most marked ventrally just posterior to the end of the nerve cord. The thickening of the hypodermis includes the dorsal and lateral portions as well as the ventral but it does not extend as far anteriorly on the dorsal side as elsewhere.

Bülow ('83), in his discussion of the normal growing region, describes this thickening, but makes no mention of any difference in the nucleoli of this region. In posterior regeneration, however, Krecker ('10) describes a marked enlargement of the nuclei and nucleoli of the ventral ectoderm. In the normal growing worm the nuclei are larger near the growing nerve cord and a short distance posterior to it than they are elsewhere (Fig. 2); the average size of these nuclei is 3.9 x 7.4 μ. The cells in which such a difference is apparent are the ones involved in the formation of the new nerve cord. The nucleoli, on the other hand, are enlarged throughout the entire tip and to a slight degree for a short distance from it. This difference is most decided in the ventral cells, in which the nucleoli may be nearly 2 μ in diameter, but average between 1 and 1$\frac{1}{2}$ μ.

Setigerous Glands.

Each pair of setæ is embedded in a mass of cells derived from the ectoderm and to them are attached secondarily mesodermal cells which form their musculature (Penners, '23). The setæ themselves are formed by the ectoderm cells, according to the description of Bergh ('90), more recently corroborated by Penners. In the earlier stages of the formation of the setigerous glands in the growing region of the tail most of the cells possess oval nuclei of about the same size as those of the neighboring ectoderm cells and nucleoli measuring between 1 and 1½ μ. As muscle fibers are added to the outside of the gland cells it becomes more difficult to study the latter but it seems certain that most of them have large nucleoli for a number of segments from the growing region. In the older part of the worm, however, the nucleoli are smaller, for the most part mere dots (Fig. 3). Occasionally a few cells are to be found possessing fairly large nucleoli (1 μ); these cells may have been active in the formation of a new seta to replace one which had been lost.

Alimentary Canal.

The cells of the alimentary canal in the first segment are typical hypodermal cells, of cuboidal shape with round nuclei 4 to 5 μ in diameter and nucleoli which appear as mere dots (Fig. 1). These cells, unlike those of the rest of the digestive tract, are without cilia but covered with a cuticle, as are those of the hypodermis (Pointer, '11). In the second somite the cells are more elongated and the nuclei are of the oval shape (3.6 x 6.9 μ) typical of most gut cells of this species. This segment is a transition zone between the first and third as far as the nucleoli are concerned. In the latter the nucleoli are fully ½ μ in diameter (Fig. 4).

These same measurements hold for both the nuclei and the nucleoli in the cells of the next few segments. At about the ninth or tenth segment, however, cells with larger nucleoli appear. These cells increase in abundance until in the vicinity of the thirteenth practically every nucleolus measures at least 1 μ (Fig. 5). There is no corresponding enlargement of nuclei. A feature which first appears in this region is the occurrence of

two nucleoli within a single nucleus. This is a phenomenon which is rather infrequent in this species except in regenerating individuals. For brevity, they will be spoken of as "double nucleoli" but this meaning of the words should not be confused with that of earlier workers, particularly Montgomery ('98), whose "double nucleoli" were individual nucleoli made up of two types of material. While no unquestionable cases of double nucleoli have been observed in the first eleven segments, posterior to this region, where the nucleoli are larger, cases of this sort are occasionally found. Counts made on several uninjured individuals indicate that the average frequency is about 3 per segment.

This larger type of nucleolus is typical of the gut cells for a considerable distance. Then there is a gradual decrease in size. In some individuals it seems to be at considerable distance from the anal segment—even 30 segments or more—while in others the larger nucleoli apparently persist at least 15 or 20 segments farther. In this posterior portion of the gut they are hardly more than mere dots in the nuclei (Fig. 2).

Mesoblasts.

In the ventral portion of the cœlom in the growing tail region, cells are present which are presumably derived from the mesoblasts of the developing individual (Wilson, '89 and '92). These cells, which produce the mesodermal structures in the newly forming segments, possess large nuclei (about 4 x 8 μ) and nucleoli (about 2 μ) (Fig. 2).

Large nucleoli are present, therefore, in the cells of the middle portion of the gut, in those of the hypodermis of the growing tail region and in the mesoblasts. The largest of these are in the cells of the ventral region of the ectoderm, which are forming the new nerve cord, and in the mesoblasts, from which the new mesodermal structures are derived. The significance of these facts will be discussed under a later heading.

Origin of New Tissue in Posterior Regeneration.

Endoderm.

In posterior regeneration in microdrilous annelids the majority of investigators, especially more recently, are agreed on the method of origin of the endodermal structures. Krecker ('10) in *Tubifex* and *Limnodrilus* removes a portion of the intestine after cutting a worm and finds that by a proliferation of cells in the old intestine growth takes place until it comes in contact with the ectoderm. He says (p. 411) that "mitosis and amitosis both occur," using as a criterion for the latter "the frequent occurrence of double and elongating nucleoli." He finds that cell division may take place one or two segments anterior to the wound, but when it "was seen at such a distance it was always found to be mitotic . . . ." His observations are verifications of statements to the same effect made by Iwanow ('03). A study of individuals which have been regenerating for various periods reveals that mitoses may and commonly do occur even eleven or twelve segments from the wound region. The distribution of mitotic figures and double nucleoli in worms regenerating for different periods is given in Tables I. to VI., inclusive.

Table I.

1 Day of Regeneration.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	8	8	7	7	10	6	4	4	5	3	2	2	2	3
Mitoses	0	0	0	0	1	0	0	0	0	0	0	0	0	0

Table II.

2 Days of Regeneration.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	25	31	20	25	21	25	23	20	22	23	19	18	6	2
Mitoses	10	8	4	3	7	4	3	3	2	5	2	2	0	0

Table III.

3 Days of Regeneration.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	26	21	34	25	27	23	25	21	23	17	9	9	7	3
Mitoses	14	2	5	2	5	3	3	0	2	5	3	1	1	0

TABLE IV.

4 DAYS OF REGENERATION.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	26	26	53	46	32	19	15	8	8	7	6	3	1	2
Mitoses	5	6	4	7	6	1	0	0	1	1	1	1	0	0

TABLE V.

5 DAYS OF REGENERATION.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	44	37	41	33	24	18	18	9	9	6	5	6	3	2
Mitoses	6	5	7	3	4	1	4	3	1	1	0	0	0	0

TABLE VI.

6 DAYS OF REGENERATION.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	20	23	18	17	20	15	16	7	5	3	4	2	1	3
Mitoses	3	2	4	1	1	0	1	0	0	0	1	0	0	0

In making these counts it is necessary to adopt some criterion for double nucleoli. These figures, therefore, are for cases where a nucleus contains two nucleoli in approximately the same focal plane of the microscope. In order that the personal equation involved in making the counts may be made as negligible as possible, instances of dumbbell-shaped or elongated nucleoli are not included. Records are not given for segments beyond the fourteenth from the wound since but 2 or 3 double nucleoli per segment, the number ordinarily found in an uninjured worm, are to be found in these. For mitotic figures all stages from the appearance of the chromosomes in the prophase to the late telophase are included.

As already mentioned, the gut cells of these worms ordinarily have nucleoli of considerable size, averaging about 1 μ in diameter, and the oval nuclei measure on the average about 4 x 7 μ. With the onset of regeneration an increase in the size of the nuclei and nucleoli begins, though it is comparatively slight in the former case.

Twelve hours after a worm is cut such a change in the nucleoli near the wound is apparent and by the second day many of them measure at least 2 μ in diameter (Fig. 6). Similarly by the second day the nuclei have enlarged until the average of a number measured is 5.1 x 8.8 μ. This increase in the amount of nuclear and nucleolar materials is not confined merely to the wound region but extends even eleven or twelve segments from it. Farther away than this, however, these structures show no change from the size ordinarily present in such a region (Fig. 5).

Double nucleoli are found in increased numbers within the same limits as are enlarged ones and beyond the twelfth or thirteenth segment from the wound they are no more abundant than in an uninjured individual (Tables I. to VI. inclusive). There is no gradual decrease in number, however, as one gets farther from the wound but a somewhat abrupt drop about the twelfth segment. Mitoses, too, are found only in these same twelve or thirteen segments and, though usually most numerous in the two or three segments nearest the bud, they too do not gradually decrease in number but stop rather abruptly.

TABLE VII.

NUMBER OF DOUBLE NUCLEOLI.

Number of Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
1	40	26	66
2	122	150	272
3	133	127	260
4	183	66	249
5	179	71	250
6	98	52	150

Double nucleoli and mitoses increase rapidly in numbers during the early part of the regeneration period and then gradually decrease again. Double nucleoli have begun to appear on the first day but only one instance of mitosis is found. There is a great increase in the number of each on the second day. By the fourth day, however, the number of double nucleoli has dropped decidedly in the segments more distant from the wound; this is counterbalanced by an increase in the five nearest segments

with the result that the total number is not appreciably changed. Table VII. gives the comparative abundance of double nucleoli in the first five segments and in the other seven which are apparently involved in the regenerative processes.

The number of mitoses also decreases in the more distant portion but in this case it is not compensated by an increase near the wound so that the total number is less. The major part of the activity of the gut in forming new tissue, therefore, is now confined to a more restricted area. Table VIII. gives data for mitoses corresponding to that given in Table VII. for double nucleoli.

TABLE VIII.

NUMBER OF MITOSES.

Number of Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
1	1	0	1
2	32	21	53
3	28	17	45
4	28	5	33
5	25	10	35
6	11	2	13

The records for the fifth day are approximately the same as those for the fourth. On the sixth the numbers both of double nucleoli and of mitoses have decreased decidedly throughout the entire region involved in regeneration. This decrease is most marked, however, in the more distant segments. By the seventh day the intestine of the old region has returned to approximately its usual appearance in an uninjured worm. Only two or three cases of mitosis are to be found and double nucleoli are little if any more abundant than in intact animals.

Beginning with the second or third day many cells in which the nucleoli are small are present in the intestine of the old part. These are probably the products of a recent mitotic division during which the nucleolus has been lost. That the nucleoli of these cells enlarge gradually is evidenced by the fact that the number of such cells does not increase appreciably during further regeneration. Similarly most of the cells in the newly formed gut tissue of the regenerating bud have small nucleoli at first.

By the third day these, too, are enlarging so that it is not possible to discover a line of demarcation between the gut of the bud and that of the old part. At about this time double nucleoli and mitotic figures begin to appear in the new gut. Cell proliferation in the new tissue thus increases as that in the old part is decreasing. Consequently, as far as the intestine is concerned, regeneration usually ceases between the sixth and seventh days. After this time it is a matter of growth in the newly formed tissue itself.

Mesoderm.

The method of formation of the new mesoderm is not as generally agreed upon as is that of the new intestine. Hepke ('97) in Naids, von Wagner ('06) in *Lumbriculus* and Abel ('02) in *Tubifex* and *Nais*, among others, maintain that the new mesodermal structures originate from ectodermal cells which migrate into the cœlom. Randolph ('92) and Iwanow ('03) in different species of *Lumbriculus* and Krecker ('10 and '23) in *Tubifex* and *Limnodrilus* find that these structures are derived from relatively unmodified cells of the mesoderm to which the term neoblasts was first applied by Randolph. Krecker ('23) reports that they are ordinarily found on the posterior surfaces of the septa in a quiescent state. After a worm is cut they enlarge to 8 or 10 times their former size and then migrate along the nerve cord to the wound region. According to his data these cells are activated on the seven septa nearest the wound with those in the four nearest segments giving the greatest response. In my preparations of individuals which have been regenerating for either three or four days a number of clearly distinguishable neoblasts are always present as far as eight or nine segments from the wound. Two examples, one of a three-, the other of a four-day regenerate, are given in Table IX. All the cells which lie upon the septa and are clearly neoblasts with nucleoli of 2 μ or over are included in these counts. All are found on the posterior surfaces of the septa and are confined almost entirely to the ventral portion. These cells correspond presumably to the later stages of metamorphosis described by Krecker ('23). In both of these cases nine segments are clearly contributing neoblasts while the tenth and eleventh are ap-

parently not involved. It is a rather common occurrence to find one or two neoblasts on a septum in an uninjured worm so that the few seen in these last segments are in accordance with what is to be expected. In making these counts, not only leucocytes, as suggested by Krecker ('23), but also nephridial cells are apt to be mistaken for neoblasts in early stages of metamorphosis. Cells of this type, as clearly demonstrated by examination of adjacent sections, are shown in Fig. 9. Both of these types of cells have quite large nuclei and nucleoli and many nephridial cells are of course in contact with the septa.

TABLE IX.

NEOBLASTS.

Segments from the wound	1	2	3	4	5	6	7	8	9	10	11
3-day regenerate	17	13	16	13	9	6	7	3	6	1	1
4-day regenerate	15	19	18	14	8	7	8	8	7	2	1

On the four or five septa nearest the wound, therefore, neoblasts are most abundant, as also observed by Krecker ('23). About nine segments in all, however, seem to be involved while Krecker reports the maximum distance as seven. The region involved in the production of neoblasts is, therefore, approximately the same as that in which cell proliferation occurs in the intestine.

Ectoderm.

The ectoderm, unlike the other two types of tissue already discussed, regenerates solely from cells in the immediate vicinity of the wound. That cells from the old hypodermis form the new hypodermis and nerve cord is generally agreed upon. Krecker ('10, p. 430) describes the marked enlargement of certain ectoderm cells "not directly opposite the nerve but somewhat dorsally, between it and the central longitudinal axis of the body." These cells increase in size and become so changed that, as he expresses it (p. 434), "were these cells seen alone they would be immediately considered neoblasts." Of the changes which take place in cells other than those of this particular region, however, he makes no more than the simple statement (p. 430) that "of course the ectoderm cells are greatly enlarged elsewhere than on the ventral side."

Twelve hours after a worm is cut the wound has healed and in those ectoderm cells which are brought into a terminal position by this process the nucleoli have begun to enlarge (Fig. 10). The cells themselves are beginning to elongate somewhat and accompanying this process the nuclei are changing to the oval shape more or less typical of elongated cells. The nuclei themselves are not appreciably enlarged at this time but the nucleoli have increased from mere dots to quite evident structures about 1 μ in diameter. Cells of this type are not confined to any definite part but make up the entire terminal portion of the hypodermis. After one day of regeneration the enlargement has gone still further but there is no marked difference in size between cells in the ventral portion and those dorsally located. The average size of the nuclei at this time is 4.5 x 7.2 μ and of the nucleoli 1.4 μ.

On the second day, however, characteristic differences make their appearance (Fig. 11). The cells which are dorsal or lateral show little if any change from the preceding day. In the median ventral region just posterior to the end of the nerve cord many of the cells have grown considerably but all gradations in size can be found between the largest of these and the cells found elsewhere in the bud. In the larger cells the nucleoli are oval and about one third the dimensions of the nuclei. The average size of ten of the larger nuclei found in two adjacent sections is 6.1 x 8.4 μ, with the nucleoli of the same cells averaging 1.9 x 2.8 μ. On the following day the nuclei of the dorsal cells are of about the same size as on the second day but many of the nucleoli have enlarged to 2 μ. In the ventral region the changes are even more marked. A number of the cells have enlarged enormously and have nuclei of about 8 x 11 μ, with nucleoli averaging about 3.5 μ. It is this stage of the hypodermal cells to which Krecker applies the term "metamorphosed" ectoderm and which may be most easily confused with neoblasts. Krecker suggests that the fact that the neoblasts come to lie near such cells is probably the reason why so many workers have thought the neoblasts to be products of the ectoderm, which seems very plausible.

Krecker ('10, p. 436) suggests, as a theory of the formation of

these much enlarged cells, that "the neoblasts have a redifferentiating effect upon the cells of the ectoderm." A close examination of the facts, however, demonstrates that the cases which he cites in proof may all be explained in another way.

In the course of regeneration the enlargement of the hypodermal cells does not appear to begin suddenly soon after the arrival of neoblasts at the wound. It seems rather to be a continuous process having its inception at the time the worm is cut. A certain time is required for the cells of the hypodermis to enlarge and those on the ventral side do not change any more rapidly than those elsewhere. They do, however, show a greater response than the others with the result that their enlargement continues after the others have slowed down. In sections of twelve-hour regenerates the ectoderm is quite different from that in uninjured individuals and the process of enlargement is clearly under way. At this time neoblasts are rarely found at the wound. Krecker ('10, p. 422) says that "twelve hours after the operation . . . in one of these (individuals) there was a neoblast at the wound, but none was migrating. In the other individual . . . two were about the wound." If it is true, then, that the enlargement of the ectoderm begins before twelve hours after the cut is made, and it certainly appears to, it is improbable that the neoblasts could have been the cause. A much simpler explanation, which seems to satisfy all of the requirements of the facts at hand, is that whatever is the underlying cause of the metamorphosis of the neoblasts on the septa is likewise the cause of the enlargement of the cells of the hypodermis at the wound. Just what may cause the neoblasts to metamorphose is not discussed by Krecker.

This view that the transformation of the ectoderm cells is independent of the presence of the neoblasts is entirely in accord with the cases cited by Krecker ('10) in proof of his theory. In one individual (p. 433) "even after three days there was no enlargement of the ectoderm. No neoblasts were about the ectoderm, in fact there was only one neoblast to be seen and this was along the nerve some distance away." He says later (p. 436) that this "exception cited in which no change in the character of the ectoderm cells occurred in the absence of the

neoblasts is of considerable significance." This case may be equally well explained in accordance with the view of an independent transformation. The factor which brings about the metamorphosis of the neoblasts and ectoderm is either absent or, because of the physiological condition of this particular individual, produces no effect. It is reasonable to suppose that, if the same underlying cause brings about like changes in these two types of cells and if they are equally susceptible to activation, one type will not undergo a process of transformation when the other does not. In all other cases given by Krecker for posterior regenerates he himself states (p. 435) that "the evidence adduced has to do only with instances in which neoblasts are found about the metamorphosed ectoderm cells." Admittedly neoblasts are present in all cases where the ectoderm cells are greatly enlarged, but an explanation for this is easily found. Neoblasts migrate only along the ventral nerve cord and so they are found in the ventral region of the bud. The ectoderm cells which are greatly enlarged are those which are to give rise to the nerve cord in the ventral region. The close proximity of these two types of cells in the regenerating bud seems to be due to the fact that both bear a definite relation to the nerve cord—those of the hypodermis to form the new portion of the nerve cord in the bud and the neoblasts using it as a pathway to the wound region. In the discussion of anterior regeneration his statement that no metamorphosis of the ectoderm and no migration of the neoblasts occurs in this type of regeneration will be considered.

The development of the setigerous glands and the production of the setæ have for the most part been neglected in the work on regeneration. Bergh ('90) and Penners ('23) both give something of a description of their formation during embryonic development. In regeneration it is difficult to observe the early stages due to the great number of cells scattered throughout the growing bud. By the fifth day the cells of the glands in the two or three segments of the bud nearest the old tissue stand out clearly since at this time the other cells of these segments have become arranged in a more orderly fashion. The nuclei of the hypodermal cells near the old tissue have begun to take on the appearance typical of the older cells. They are

oval—measuring about 3.5 x 6 μ—and contain nucleoli less than 1 μ in diameter. The nuclei of these gland cells, however, have not decreased in size but are about the same as those of the enlarged ectoderm cells of the dorsal and lateral regions (Fig. 12). The average measurement of twenty-five of these is 5.2 x 7.3 μ with nucleoli 1.8 μ in diameter. Occasionally double nucleoli are to be found. The fact that these cells push into the cœlom offers another point of confusion which may have led early workers to think that the ectoderm migrated into the cœlom to form the new mesodermal structures.

It will be seen that in all three types of cells (endodermal, mesodermal and ectodermal) which take part in the formation of new tissue at the posterior end there is one outstanding feature in common. The nuclei and nucleoli enlarge. The greatest changes occur in the neoblasts and in the cells of the ventral portion of the hypodermis. In both of these types the nuclei of the fully transformed cells are eight or nine times as large as ordinarily. The nucleoli increase even more in proportion, particularly in the hypodermal cells in which they enlarge from mere dots to structures over 3 μ in diameter. Even in the gut cells where the nucleoli are usually of considerable size there is probably a ten-fold increase. The significance of these facts will be discussed later.

Origin of New Tissue in Anterior Regeneration.

Endoderm.

In anterior regeneration as in posterior regeneration the majority of investigators—Rievel ('96), Haase ('98), von Wagner ('00), Iwanow ('03) and Krecker ('10)—are agreed that most of the intestine of the regenerating bud is formed by the growth of that in the old part. This formation of new tissue involves about eleven or twelve segments here as in posterior regeneration. The nucleoli are considerably enlarged at the end of the first day and double nucleoli are fairly common. Only a few mitoses, however, are present. The numbers both of double nucleoli and of mitoses reach a maximum between the second and third days. On the fourth day both are fewer in number in the more distant segments. In the case of double nucleoli this decrease is partially

compensated by an increase in the region nearest the wound. Mitoses, however, are somewhat less frequent in this region too. As in posterior regeneration the numbers continue to decrease and by the seventh day both of these features are rare in the gut of the old part. Tables X. and XI. give records for two- and four-day regenerates, respectively. Tables XII. and XIII. give comparative data for the nearer and more distant segments for double nucleoli and mitoses, respectively.

There is little, if any difference, then, in the behavior of the cells of the intestine in anterior and in posterior regeneration.

TABLE X.

2 DAYS OF REGENERATION.

Segments from the wound.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	27	26	21	27	34	30	30	33	32	29	16	16	1	1
Mitoses.....	12	6	3	7	8	4	3	4	2	3	2	0	1	0

TABLE XI.

4 DAYS OF REGENERATION.

Segments from the wound.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	38	31	29	32	34	26	14	7	8	6	2	3	3	1
Mitoses.....	12	4	3	4	7	3	1	0	1	1	0	0	1	0

TABLE XII.

DOUBLE NUCLEOLI.

Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
2	135	186	321
4	164	66	230

TABLE XIII.

MITOSES.

Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
2	36	18	54
4	30	7	37

Mesoderm.

Many investigators—Hepke ('97), von Wagner ('00) and Abel ('02) among others—are of the opinion that the mesoderm both of anterior and of posterior regenerates is formed from the ectoderm. The weight of evidence in posterior regeneration, as previously mentioned, now seems to favor mesoderm formation from neoblasts. In anterior regeneration Iwanow ('03) in *Lumbriculus variegatus* and Krecker ('10) in *Tubifex* and *Limnodrilus* find that the new mesoderm is formed from the old mesoderm and not from regeneration cells as in posterior regeneration. They find a distinct fraying out of the longitudinal muscles at the wound. Many of the cells then lose their contractile substance and wander about in the cœlom where they become mixed with other cells—peritoneal, connective tissue and ectodermal—so that it is "hard to distinguish between the various types" (Krecker, '10, p. 441). After about the fifth day of regeneration they begin to develop contractile substance and to become arranged in muscle masses. The production of the body musculature is much slower in anterior than in posterior regeneration. "In specimens killed three weeks after the operation the musculature of this region was still in a very undeveloped state" (Krecker, '10, p. 442).

As the matter stands at present, then, investigators are divided into two groups regarding the formation of the new mesoderm. Those in one believe that it is derived from the ectoderm, those in the other that it is produced by a partial dedifferentiation followed by a redifferentiation of the cells of the old mesodermal structures. Those who support an ectodermal origin of the mesodermal structures believe that the mesoderm is formed in the same manner at both ends. Those of the other group, however, think that the origin is due to one type of mesodermal cell at the posterior end—the neoblasts—and to another at the anterior end—the specialized cells which dedifferentiate.

In anterior regeneration, just as in posterior regeneration, there are many opportunities for confusion. In the former it is even more difficult than in the latter to follow just what happens. The migration of ectoderm cells into the cœlom in the formation of the cerebral ganglion might easily mislead one. In addition

to this the presence of many other cells of similar appearance make it practically impossible to say, from an examination of fixed specimens, just what cells do form the various tissues. Since in posterior regeneration it is quite apparent that ectoderm is not involved in the formation of the septa or longitudinal muscles it seems reasonable to assume that it does not take part here. This view is supported further by the fact that cells of mesodermal origin are present in the bud.

Regarding the conception that there is a partial dedifferentiation of the old muscle cells which then form the longitudinal musculature of the bud, there seems to be conflicting evidence. These cells certainly do fray out and some of them seem to lose their contractile substance in the segment injured by the cut. Such a behavior is not, however, peculiar to the anterior end in *Lumbriculus inconstans*. Just as other cells are affected by the cut so many muscle cells are dislodged or injured. It is not improbable that such cells should then lose their contractile substance due to the injury; such a change might, therefore, be a step on the road to destruction rather than on that to repair. The fact that this same sort of change does take place at a posterior cut-surface, where the muscle cells are not involved in the regenerative processes, seems to support this view. The migrating spindle-shaped cells, which appear between the first and second days in *Lumbriculus*, are clearly derived from the hypodermis (Fig. 13). These cells migrate into the cœlom and there form the cerebral ganglion. It may perhaps be this type of cell which has been observed by Krecker in *Tubifex* and *Limnodrilus*.

Further evidence is derived from a study of neoblasts in anterior regenerates. Krecker finds that in the species with which he worked they are usually not activated at all posterior to a cut and that resting neoblasts may be found on a nearby septum. "The individuals upon which these observations were made were all killed three weeks or more after the operation so that the failure of the neoblasts to act as they do at the posterior end could hardly have been due to lack of time" (Krecker, '10, p. 437). In the individuals used in his experiments—of the genera *Tubifex* and *Limnodrilus*—anterior regeneration does not

take place at the level of the twentieth somite where these cuts were made. It does not seem strange, therefore, that at the end of three weeks all neoblasts should be resting even if activation had taken place three weeks previous. In *Lumbriculus* anterior regeneration does take place at all except very posterior levels. The amount of tissue is, of course, limited to a few segments, usually five or six, rarely seven (von Wagner, '00).

Counts made of the number of clearly recognizable neoblasts (intermediate and definitive stages of Krecker, '23) present on the various septa in three- and four-day regenerates are given in Table XIV. These cells all possess nucleoli of approximately 2 μ or over (Fig. 14).

TABLE XIV.

NEOBLASTS.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11
3-day regenerate	6	21	14	9	11	13	3	3	4	2	1
4-day regenerate	16	14	23	13	7	8	2	6	3	1	1

These figures do not differ greatly from those for posterior regenerates (Table IX.). The small number of neoblasts present on the first septum of the three-day regenerate may be explained in part by the fact that this septum was partially torn away by the cut in this particular case. About four segments seem to produce neoblasts more abundantly than the rest and about nine segments in all are apparently involved. The more active region extends farther, however, in the three-day regenerate; the inclusion of the first septum in the cut brings this about. The neoblasts, then, are activated to about the same extent as at the posterior end. The number present in the bud at the end of two days, however, indicates that either not as many migrate anteriorly or else the migration is much slower and the cells are used up as they get to the wound. No mass of large cells is to be found in the ventral part but several cells are usually present and a number can generally be found migrating along the nerve cord (Fig. 15). The apparently slow migration of the neoblasts anteriorly is perhaps the reason why the mesoderm in the newly formed head does not show signs of development until after the

fifth day (Krecker, '10). After the third day it is difficult to say just what does happen in the bud region. Many metamorphosed ectoderm cells are present and things are so confused that it is difficult to distinguish the various types of cells, particularly those as similar in appearance as are the neoblasts and the much enlarged cells from the ventral hypodermis.

The process of formation of new septa and longitudinal muscles by cells known to be derived from neoblasts has not been observed. The fact that neoblasts metamorphose in considerable numbers and are frequently seen migrating along the nerve cord is, nevertheless, evidence which seems to indicate that they play some part in anterior regeneration in *Lumbriculus*. If such is the case, the mesodermal structures are formed in the same manner in both types of regeneration, just as are the ectoderm and endoderm.

Ectoderm.

As in posterior regeneration only those cells in the immediate vicinity of the wound take part in the formation of the new ectodermal structures. There occurs a similar enlargement of all these cells during the first day or so, no one region undergoing any more extensive change than any other. Iwanow ('03) in his discussion of the formation of the new nerve elements at the anterior end describes a marked enlargement of the ectoderm cells. Krecker ('10, p. 433) says that the ectoderm cells "undergo no such metamorphosis" as at the posterior end, explaining this on a basis that no neoblasts migrate anteriorly to stimulate it to activity. As in the case of posterior regeneration, however, by the second day there are cells on the ventral side which have quite large nuclei and nucleoli, and by the third day they have reached a considerable size (Fig. 16). The area over which this marked enlargement extends is greater than in posterior regeneration. Large nuclei are present in cells found over a considerable portion of the ventral and ventro-lateral hypodermis and even somewhat dorsally in the terminal portion. The largest cells, nevertheless, are confined for the most part to the ectoderm in the vicinity of the mid-ventral line. This region extends from the end of the old nerve cord anteriorly. The nuclei and nucleoli measure about the same as in the transformed cells of the

regenerating tail (8 x 11 μ and 3.5 μ, respectively). In the dorsal portion of the terminal hypodermis many of the cells are apparently in the process of breaking away from the epithelium to migrate into the cœlom where they form the cerebral ganglion (Fig. 13). They become elongated and slender in this process and develop an appearance which answers well the description of cells which Krecker ('10, p. 441) believes are mesoderm cells migrating anteriorly to form the new longitudinal muscles—"spindle-shaped cells with slightly granular cytoplasm and large nucleus containing a deeply staining nucleolus."

Regarding the activation of the ectoderm by the presence of the neoblasts, there is no further evidence from anterior regeneration. Neoblasts apparently migrate anteriorly and the ectoderm is also transformed, similar processes to those occurring at the posterior end.

From these observations it is apparent that not only the ectodermal and endodermal elements of the regenerated head are derived in the same manner as at the posterior end but the mesodermal structures as well. Just as in posterior regeneration the neoblasts apparently metamorphose and migrate to the wound region and the cells of the ventral portion of the hypodermis become greatly enlarged. About ten or eleven segments seem to be involved in the regenerative processes except in the case of the ectoderm in which the changes are confined to the immediate vicinity of the wound as in posterior regeneration.

Discussion.

The production of new tissue in the anterior regeneration of microdrilous annelids seems to be essentially the same as in the posterior regeneration in these forms. Iwanow ('03) in *Lumbriculus variegatus* and Krecker ('10) in *Tubifex* and *Limnodrilus* describe the formation of the new mesodermal tissue from neoblasts at the posterior end but believe that there is a dedifferentiation of old mesoderm to form the new at the anterior end. The loss of contractile substance by some of the longitudinal muscle cells at the cut surface, however, occurs at both ends. The spindle-shaped cells abundant in the dorsal part of the bud in anterior regeneration may be seen in *Lumbriculus inconstans*

to be derived from the hypodermis, migrating into the cœlom to form the cerebral ganglion (p. 295). The fact that many neoblasts metamorphose and migrate anteriorly is evidence that they probably take part in the building of the new mesoderm in anterior as well as in posterior regeneration. The presence in the bud of other cells of similar appearance makes it practically impossible to follow the laying down of these structures from cells of known origin. It has for some time been agreed by investigators that both endodermal and ectodermal structures are formed in the same manner in both types of regeneration, that is, from the old intestine and body wall, respectively. It seems evident then that the mesodermal structures are produced in the same way at both ends and are not an exception as previously believed.

An explanation for the formation of the new mesoderm from more or less undifferentiated mesodermal cells rather than from the muscles may perhaps be found in the fact that the cytoplasm of the muscle cells is highly modified. While the cells of the hypodermis and intestine are of a simple, cuboidal or columnar shape, the development of the contractile substance by the muscle cells brings about an extensive modification of the cytoplasm of these cells. Consequently, cells from the peritoneum, less modified than the others are called upon to form the tissue in the regenerating bud. The development of the new nerve cord is a process not essentially different. In this case the cytoplasm is considerably modified in the formation of fibers and, instead of the nerve cord near the wound dedifferentiating to produce the new tissue, the hypodermal cells of the ventral side are called upon to furnish the new material. While these facts may not necessarily indicate that one type of cell is any "more differentiated" than another, the muscle and nerve cells in *Lumbriculus* certainly are less susceptible to the activating stimulus than are those of the other tissues.

In regeneration the ectoderm cells enlarge considerably especially those in the median ventral line which may develop nuclei as large as $8 \times 11\ \mu$ with nucleoli between 3 and $4\ \mu$ in diameter. Krecker ('10 and '23) is of the opinion that this change in the ventral cells is due to "some influence" of the

neoblasts. Evidence presented here (pp. 290 and 297) does not support this idea but rather the view that a similar underlying cause brings about the changes which occur in the metamorphosis both of the neoblasts and of the ectoderm cells. During the first two days of regeneration all the cells of the hypodermis in the immediate vicinity of the wound develop large nuclei and nucleoli. After this time the ones found dorsally and laterally slow down whereas those in the ventral region continue to enlarge. Neoblasts are rarely, if ever, found at the wound at the time when the increase in size of the hypodermal cells becomes apparent (between six and twelve hours after the cut). From that time until the largest cells are formed the enlargement seems to be a continuous process without any sudden change or increase in rate which might be produced by the presence of neoblasts. The fact that in posterior regeneration metamorphosed ectoderm cells and neoblasts are always found very near one another is cited by Krecker ('10) as proof that there is some relation between them. This proximity of these two types of cells is perhaps equally well explained if the reason for the presence of each in the ventral region is considered. The cells of the ventral hypodermis are transformed in the process of proliferation and migration to form the nerve cord of the regenerating region. The neoblasts migrate to the posterior end from the old tissue along this same structure. These cells, then, are brought together not because one causes the modification of the other, but rather from the fact that each type has a certain relation to the nerve cord. The view that the same cause produces the changes in the cells of both types seems to fulfill all the requirements and to be a simpler explanation than that given by Krecker. Furthermore, there is no apparent reason why the ectoderm, any more than the endoderm, should need to be activated by another type of cell.

Function of the Nucleolus.

Various theories have been advanced regarding the function of the true nucleolus or plasmosome. Montgomery ('98) and Ludford ('22) give a rather complete discussion of the work of many investigators. The more recent results seem to indicate

that the nucleolus bears some relation to the activity of the cell body and nucleus. Many cases have been reported of nucleolar extrusions preceding the formation of yolk granules. Ludford ('22) also reports that in the more active cells of the endoderm of *Limnæa* the nucleoli are larger than in those of the hypodermis. He is "inclined, therefore, to regard the size of the nucleolus as an indication of the degree of metabolism existing in the cells—the greater the metabolic activity, the larger the total volume of nuclear (nucleolar ?) matter present in the nucleus, or extruded into the cytoplasm" (p. 139). Wilson ('25, p. 96) also believes that there is a "question whether the nucleolus may not play a more active and important part in cell metabolism than most writers have hitherto assumed."

An examination of slides of *Lumbriculus*, both of uninjured and of regenerating individuals reveals no cases of nucleoli which could be interpreted as being extruded into the cytoplasm from the nucleus. There is considerable evidence, however, that nucleolar size is in some manner an indication of the degree of metabolic activity of the cell.

In uninjured worms the gut cells of the first eleven or twelve segments have small nucleoli. Similarly those for some distance from the anal opening have a comparatively small amount of nucleolar substance. In the intermediate portion, however, relatively large nucleoli are present. The cells of the mouth cavity and esophagus naturally do not take as great a part in the digestive processes as do those found more posteriorly. In the mid-gut the digestive fluids are being poured into the lumen and the food is being absorbed. Consequently considerable cell activity is necessary. Toward the posterior end such activity naturally drops off again. The size of the nucleoli, therefore, parallels more or less the extent of the activity expected of the cells in the various regions of the gut (p. 281).

Furthermore, in the case of the setigerous glands, the nucleoli of the cells are large in the growing tail region and in a regenerating bud where the new setæ are being rapidly formed (pp. 281 and 291). In the old segments of a worm, however, where the setæ have been present for a considerable time, the nucleoli are usually small (p. 281). The cells in the active

portion of the nephridia possess nucleoli of considerable size, too. In fact, these cells are very similar in appearance to intermediate neoblasts, from which they may be distinguished by the presence of large granules in their cytoplasm (p. 288). Similarly, as described by Krecker ('23), the phagocytes have very large nucleoli.

Again, in the growing tail, just as in the regenerating individual, the hypodermal cells have enlarged nucleoli, particularly on the ventral side where the nerve cord is being formed (p. 280). Those cells which are forming the new mesoderm—presumably derived from the primary mesoblasts in the embryonic development (Wilson, '89 and '92)—also have very large nucleoli, differing very little in appearance from the neoblasts in a regenerating tail (Randolph, '92). In contrast to this, in the older part of the worm, the nucleoli of the mesoderm and ectoderm cells are very small, in the case of the latter mere dots under a magnification of 1,000 ×.

In regeneration, as already mentioned, the nucleoli of the neoblasts and hypodermal cells become greatly enlarged. There is also at this time an increase in the amount of nucleolar substance in the gut cells. For ten or eleven segments from the wound, the nucleoli enlarge, a process followed by the appearance of numerous instances of double nucleoli—two within a single nucleus (p. 285).

This occurrence of double nucleoli is taken by Iwanow ('03) and Krecker ('10) as evidence that amitosis is frequent in the production of the new gut tissue. In *Lumbriculus* there is no evidence of any division or even of a clearly defined constriction in any of the nuclei of the gut which contain two nucleoli. The individual nucleoli in the case of the double one are usually smaller, and in no case larger, than those in the neighboring cells where but a single nucleolus is present. It seems, then, that the division of the nucleolar material into two parts is not in preparation for a succeeding cell division. Rather as this material accumulates it continues to exist in a single droplet until it reaches a certain size and then divides. This splitting into two parts may perhaps be due to the fact that a droplet of material of its consistency and composition has a certain

maximum size beyond which it cannot exist as an individual droplet under the conditions existing in the nucleus. This view is in accord with our knowledge of the limitations to drop size in emulsions.

After one day of regeneration many cells show this increase in the amount of nucleolar material, as evidenced by the occurrence of a number of double nucleoli as well as the enlargement of the single ones. On the second and third days the frequency of double nucleoli reaches a maximum and after that time falls off slowly. Following the increase in nucleolar material, there appear numerous cases of mitosis. It seems probable that this increase in nucleolar substance is indicative of a heightened activity on the part of the cells in preparation for cell division.

A comparison of the amount of nucleolar material in the various types of cells taking part in regeneration reveals a distinct parallelism between this amount and the relative activities of these cells. There are four general types of cells involved: (1) those of the old gut which form the new gut; (2) those of the old dorsal and lateral hypodermis which build the new hypodermis; (3) those of the old ventral hypodermis of which the special function is to furnish the material for the nerve cord in the regenerating bud; finally (4) the neoblasts which form the new mesodermal structures. Of these, the cells of the first two types maintain to a certain extent their usual epithelial arrangement, only a comparatively small amount of migratory activity occurring. Their nucleoli enlarge considerably but by no means as much as in the case of the other two types. The ventral ectoderm cells and the neoblasts, when fully transformed, are of about the same general size and appearance and have nucleoli of nearly twice the diameter of those in the other cells. The ventral ectoderm cells must naturally undergo rapid proliferation to supply all of the material necessary for the nerve cord; the neoblasts must migrate to the wound region and there multiply with considerable rapidity. There is some relation in *Lumbriculus*, then, between the functional activity of the cells and the amount of nucleolar material present in them. This is a conclusion similar to that drawn by Ludford ('22) from a study of *Limnæa* with particular reference to the behavior of the nucleolus in oögenesis and cleavage.

Summary.

Nuclei and Nucleoli in Uninjured Individuals.

1. The nuclei and nucleoli of the hypodermal cells are small except in the growing tail region. Here they are enlarged, especially in the cells on the ventral side which are involved in the formation of the new nerve cord.

2. Large nucleoli are present in the cells of the setigerous glands near the growing region of the tail. In old segments they are small.

3. The gut nucleoli are small in the first twelve segments. They are larger from this region up to twenty or thirty segments from the posterior end. In these segments, they are again small.

4. Double nucleoli are occasionally found in the mid-gut, where large nucleoli are present.

Origin of New Tissue in Regeneration.

5. Double nucleoli and mitoses are found in the intestine for eleven or twelve segments from the wound. In this same region the nucleoli are considerably enlarged.

6. Cell proliferation in the old intestine practically ceases between the sixth and seventh days of regeneration.

7. Neoblasts metamorphose and migrate to the wound at the anterior end as well as at the posterior end. At least eight or nine segments furnish these cells, the four or five nearest the wound apparently playing the most important part as observed by Krecker.

8. The failure of the muscle and nerve cells of the old part to form the corresponding new structures in regeneration is perhaps due to the fact that the cytoplasm of these cells has become highly modified, thus rendering them less susceptible to activation.

9. The spindle-shaped cells in the dorsal portion of the bud cavity at the anterior end are derived from the hypodermis and not from the muscles of the old part.

10. In both anterior and posterior regeneration the nuclei and nucleoli increase in size in the ectoderm cells in the immediate

vicinity of the wound. This enlargement is no more rapid in one part than in another; it continues longer in the ventral cells so that by the second day it is greater there.

11. The metamorphosis of the ectoderm is not in all probability due to the proximity of the neoblasts, as supposed by Krecker, but instead to an independent transformation.

12. The cells of the setigerous glands in the new bud have large nuclei and nucleoli.

13. A feature common to all the cells which take part in the formation of the tissues in the regenerating bud is the presence of large nuclei and nucleoli.

14. The amount of nucleolar material present in a cell seems to be an index of the activity of its nucleus both in cell-metabolism and in preparation for cell division.

15. In *Lumbriculus* there is no evidence of any division or even of a clearly defined constriction in any of the nuclei of the gut which contain two nucleoli.

16. The presence of two nucleoli in a single nucleus is not a step in amitosis, as many have supposed, but is due to the increase in nucleolar substance beyond the amount which can exist within that particular nucleus as a single droplet.

17. The various tissues seem to be derived in the same manner both in anterior and in posterior regeneration.

Conclusions.

1. Both in anterior and in posterior regeneration the mesoderm is formed from neoblasts. Iwanow and Krecker are in error in the belief that cells from old specialized mesodermal structures form the new ones in the anterior regeneration of *Lumbriculus*.

2. There is a certain predetermined area of the hypodermis on the ventral side which metamorphoses preparatory to the formation of new nervous tissue during regeneration. The cells of this region are probably activated by the same stimulus as are the neoblasts. Krecker's view that the neoblasts have an inciting effect on the cells of this region seems unfounded.

3. The amount of nucleolar material present in a cell seems to be an index of the activity of its nucleus both in cell-metabolism and in preparation for cell division. Two nucleoli within a single

nucleus are the result of an increase in nucleolar substance beyond the amount which can exist within the nucleus as a single droplet. Their presence is not a step in amitosis.

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KEY TO PLATES.

All figures are photomicrographs. With the exception of Fig. 11, all were taken with a Zeiss apochr. 2 mm., eyepiece 10; these are magnified 640 ×. For Fig. 11, a B. and L. apochr. 4 mm. and eyepiece 6 were used; this is magnified 290 ×. The following are the symbols used in the figures:

a., anal opening,
c., cuticle,
chl., chloragogue cells,
d. e., enlarged dorsal ectoderm cells,
d. n., double nucleoli,
g., gut,
gl., gland,
h., hypodermis,
m., mitosis,
m. c., mouth cavity,
m. e., metamorphosed ectoderm cells,
mes., mesoblast,
migr., migrating ectoderm cell,
n., nucleus containing nucleolus,
neph., nephridial cell,
nb., neoblast,
nv., nerve cord,
s., seta,
sep., septum.

PLATE I.

Uninjured Individuals.

FIG. 1. Mouth cavity and hypodermis, showing small nuclei and nucleoli in this region.

FIG. 2. Growing region at posterior end.

FIG. 3. Setigerous gland of an old segment.

FIG. 4. Gut of fourth segment.

FIG. 5. Gut of thirteenth segment.

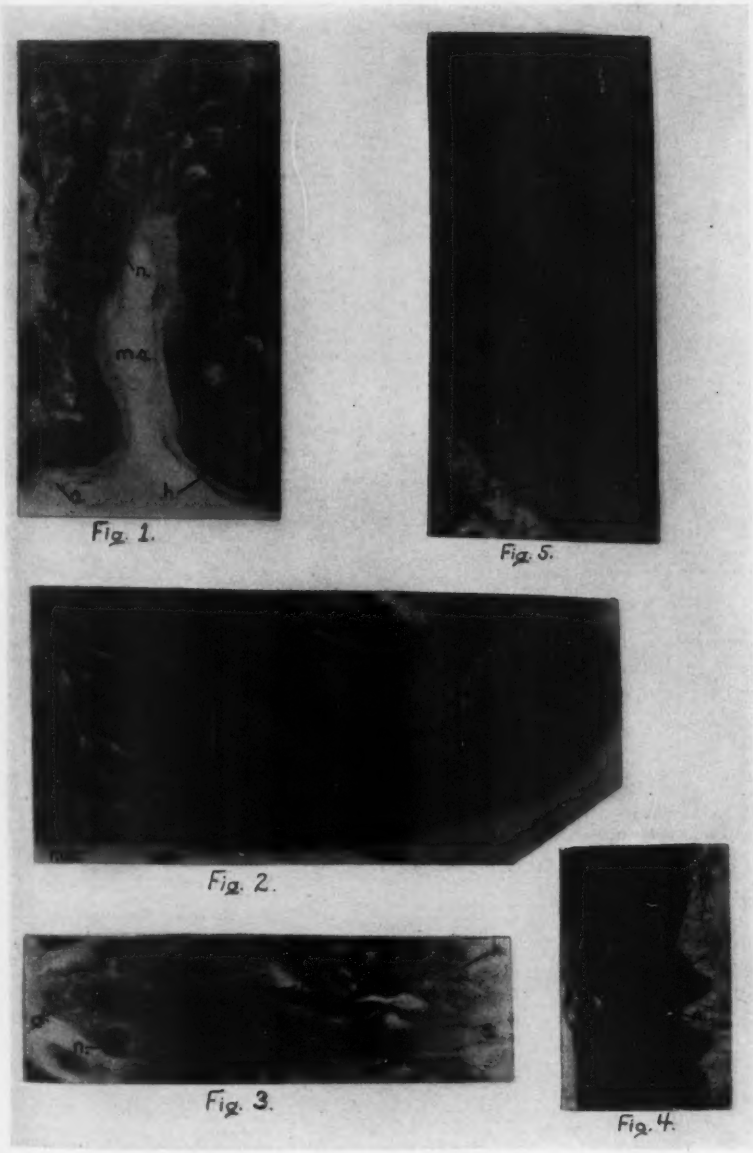
n.
m.a.
h
Fig. 1.
Fig. 5.
Fig. 2.
Fig. 3.
Fig. 4.

PLATE II.

Regenerates.

FIG. 6. Two-day posterior regenerate. Gut cells of second segment from wound.

FIG. 7. Same individual as in Fig. 6. Gut cells of tenth segment from wound.

FIG. 8. Same individual as in Fig. 6. Double nucleoli in gut cells of fourth segment from the wound.

FIG. 9. Three-day anterior regenerate. Nephridial cells in a position in which they might be mistaken for neoblasts. Arrow points toward the anterior end.

FIG. 10. Twelve-hour posterior regenerate. Enlarging ectodermal cells in wound region.

FIG. 11. Posterior regenerating bud at the end of two days.

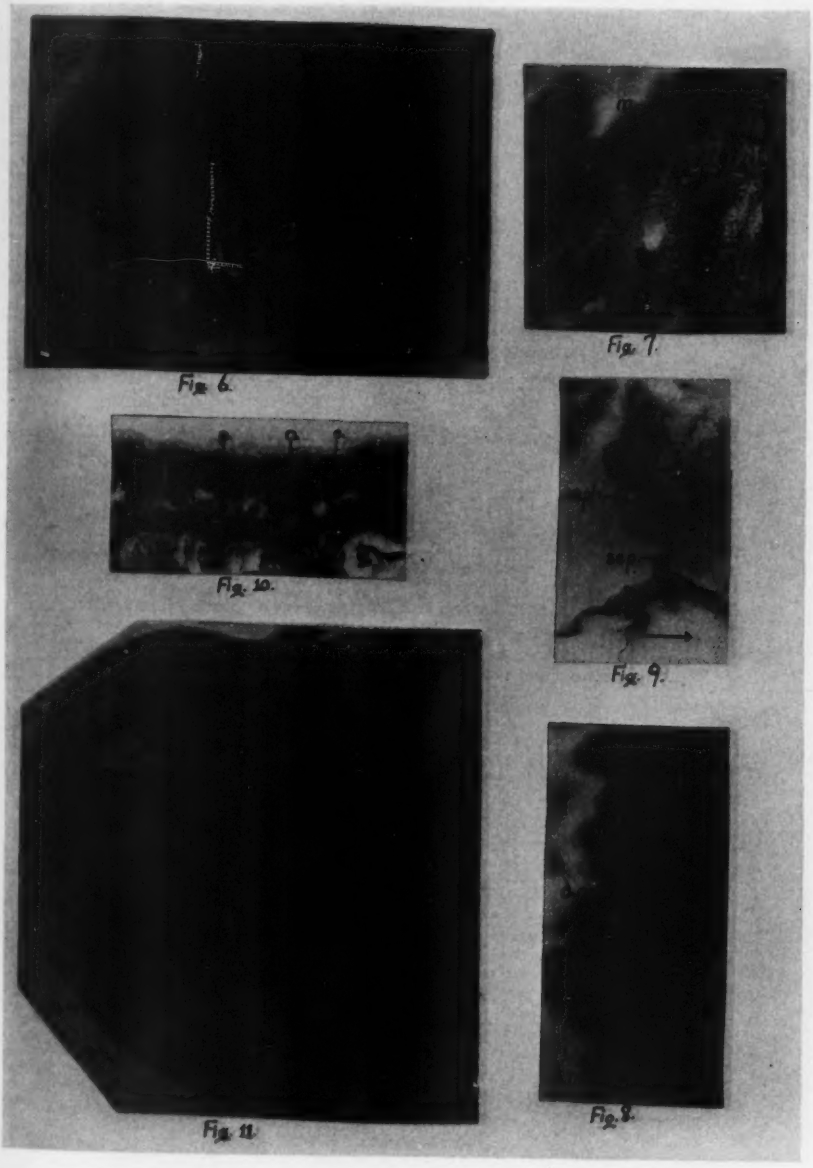

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PLATE III.

Regenerates.

FIG. 12. Six-day posterior regenerate. Setigerous glands producing new setæ.

FIG. 13. Two-day anterior regenerate. Spindle-shaped cells of ectoderm migrating into bud cavity.

FIG. 14. Two-day anterior regenerate. Neoblasts metamorphosing on the posterior surface of the fifth septum from the wound. Arrow points toward anterior end.

FIG. 15. Two-day anterior regenerate. Neoblasts at anterior end of nerve cord.

FIG. 16. Three-day anterior regenerate. Metamorphosed cells of ventral ectoderm.

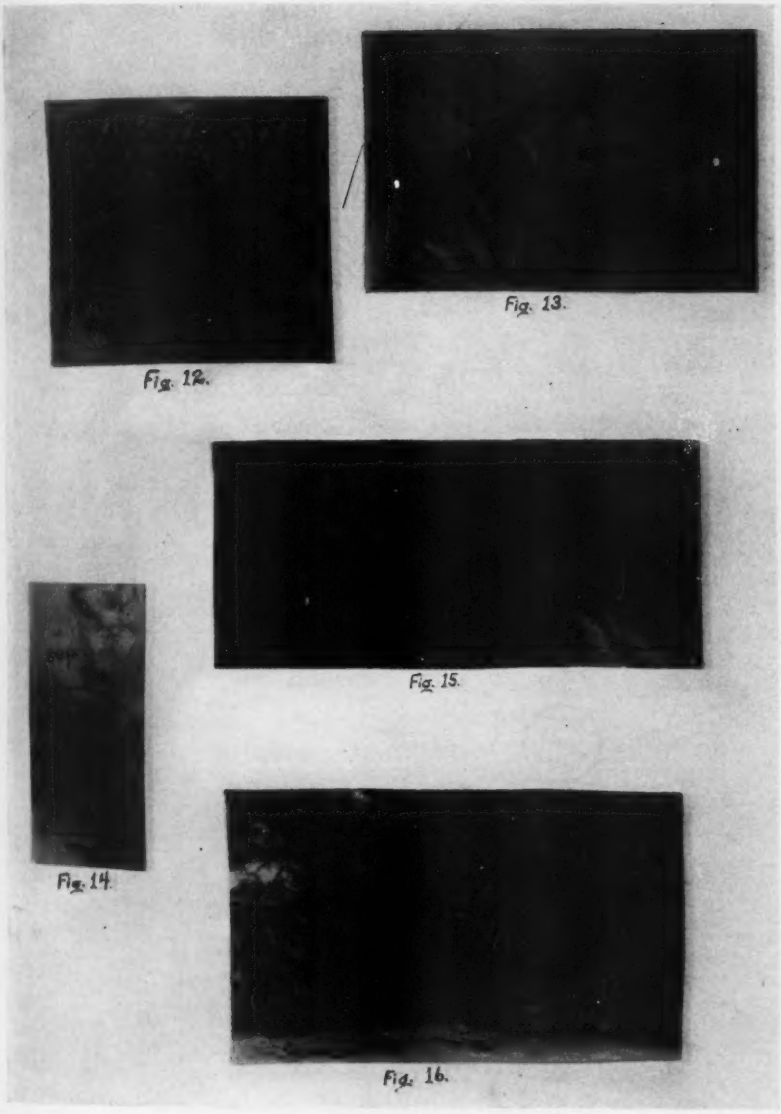

Fig. 12.

Fig. 13.

Fig. 15.

Fig. 14.

Fig. 16.